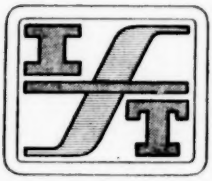
Food Research

An Official Publication of

The Institute of Food Technologists



IN THIS ISSUE

Methods of Grading Pectin in Relation to the Molecular Weight (Intrinsic Viscosity) of Pectin

Significance of Variations in Observed Slopes of Thermal Death Time Curves for Putrefactive Anaerobes

Effects of Light on the Formation of Carotenoids in Tomato Fruits

Raw Starch Digestion: A Comparison of the Raw Starch Digesting Capabilities of the Amylase Systems From Four Alpha-Amylase Sources

Denaturation of Myosin During Defrosting of Frozen Fish

Errors of the Second Kind in Organoleptic Difference Testing

The Lead, Zinc and Copper Content of Foods

Amino Acid Composition of Organ Meats

Nutritive Value of Milk Proteins. II. Stability During Sterilization of Evaporated Milk as Determined by the Rat Growth Method

Composition of Certain Species of Fresh-Water Fish. I. Introduction: The Determination of the Variation of Composition of Fish

Biuret Method for Soluble Whey Proteins in Nonfat Dry Milk Solids

Ropy Milk. I. The Biology of Rope Production

Ropy Milk. II. The Influence of Physicochemical Environment of the Formation of Ropy Substance

Ropy Milk. III. Chemical Studies of Ropy Substance

Comparison of the Nutritive Values of Mono-, Di-, and Triglycerides by a Modified Pair-Feeding Technique

Volume 19 MARCH-APRIL, 1954 Number 2

Food Research

An Official Publication of
The Institute of Food Technologists

Volume 19 MARCH-APRIL, 1954 Number 2

EDITORIAL BOARD

MARTIN S. PETERSON, *Editor-in-Chief*
(Editorial Office: 11606 South Bell Avenue,
Chicago 43, Illinois)

J. GRANT ARMSTRONG
A. K. BALLS
J. S. BLAIR
KENNETH W. BRIGHTON
G. M. DACK
R. R. LEGAULT
JOHN R. MATCHETT
PAULINE PAUL
LOUIS B. ROCKLAND
ROBERT G. TISCHER
JAMES RICHARD VICKERY
O. B. WILLIAMS

FRED W. TANNER, *Editor Emeritus*
Z. I. KERTESZ, *Consulting Editor*

CHARLES S. LAWRENCE, *Business Manager*
176 West Adams Street
Chicago 3, Illinois

Published by the Institute of Food Technologists six times a year. Printed by The Garrard Press, 119-123 West Park Avenue, Champaign, Illinois. Entered as second-class matter at the Post Office at Champaign, Illinois, under the act of March 3, 1879. Accepted for mailing at special rate of postage provided for in amended Section 34.40(e), pursuant to the provisions of Public Law 233, approved October 8, 1951.

INSIDE SCIENCE

White Flour in the U.S.A.—

Enriched* with Vitamins and Iron for Better Nutrition

by Science Writer

This article is one of a series devoted to the enrichment of family white flour, white bread and rolls, corn meal and grits, macaroni products and white rice.

The enrichment of family white flour with thiamine, riboflavin, niacin and iron by American millers is a major success. It has made great contributions to better health in the United States by improving a staple food.

Since the start of the program in 1941 by millers, voluntarily, the evidence is conclusive that enrichment not only has reduced the number of cases of certain dietary diseases but also is promoting the mental and physical vigor and well-being of the U. S. population generally. Because of its demonstrated value, the principle and practice of enrichment have been applied to other foods made from grains: corn meal and grits, white rice, macaroni, spaghetti, noodles, pastina, farina—and, of course, to white bread and rolls.

Doctors and diet experts have long supported white flour enrichment. The Council on Foods and Nutrition of the American Medical Association and the Food and Nutrition Board of the National Research Council are on record as endorsing the practice.

The legislatures of a majority of the States plus Hawaii and Puerto Rico have enacted laws which make mandatory the enrichment of all family white flour, as well as white bread, sold commercially in those areas.

American homemakers, too, favor foods they know to be enriched—a fact demonstrated by surveys. They look for these words on package labels: "Enriched with vitamins and iron for better nutrition."

What Is Enrichment?

It's an axiom in the milling industry that consumers want beautifully fine, white flour. When wheat is milled and processed to get the white flour which the public demands, vitamin and mineral values are unavoidably lost.

Enrichment restores to white flour the following important vitamin and mineral factors: thiamine, riboflavin, niacin and iron. Calcium also may be added as an optional ingredient. The process is simple and inexpensive. A mixture of the vitamins and iron is fed into the flour stream during processing. This insures that the enriching ingredients are spread evenly throughout the flour.

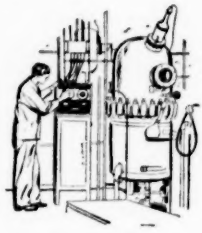
The U. S. Food and Drug Administration has established standards which white flour must meet to be properly labeled enriched. The requirements, in milligrams per pound, are:

	Min.	*Max.*
Thiamine (vitamin B_1)	2.0	2.5
Riboflavin (vitamin B_2)	1.2	1.5
Niacin (another "B" vitamin)	16.0	20.0
Iron	13.0	16.5

(In Canada, too, the same standards have been set for enriched white flour through the amendment of the Food and Drugs Act.)

Vitamins Are Made

The science of chemistry is so advanced these days that many of Nature's complex substances can be "duplicated" in the laboratory. This has happened with many vitamins. First, the chemical composition is learned. Second, the pure substance is isolated. Third, a "duplicate" is made by synthesis. And fourth, the laboratory techniques are extended to large scale operation.



The manufactured "duplicate" is identical chemically and in biological activity with Nature's own product. A vitamin is still a vitamin regardless of its source. So efficient is large scale manufacturing that vitamins are sold at a lower cost than if they were extracted from natural sources.

The Hoffmann-La Roche people make top quality vitamins actually by the tons. To do this they use amazingly complex processes with scientific production controls and the latest equipment which fill buildings each a city block square and many stories high.

Reprints of this article, and all others in this series are available without charge. Please send your request to the Vitamin Division, Hoffmann-La Roche Inc., Nutley 10, New Jersey. In Canada: Hoffmann-La Roche Ltd., 286 St. Paul Street, West; Montreal, Quebec.

The watermills are gone. Today's needs require today's methods. How sensible it is that millers across the nation restore health-giving vitamins and minerals through enrichment.

*Webster's Merriam Collegiate Dictionary includes this definition of "enrich": "to improve (a food) in nutritive value by addition in processing of vitamins and minerals".

Volume 19 MARCH-APRIL, 1954 Number 2

CONTENTS

METHODS OF GRADING PECTIN IN RELATION TO THE MOLECULAR WEIGHT (INTRINSIC VISCOSITY) OF PECTIN. Povl E. Christensen 163

SIGNIFICANCE OF VARIATIONS IN OBSERVED SLOPES OF THERMAL DEATH TIME CURVES FOR PUTREFACTIVE ANAEROBES. Arthur M. Kaplan, Howard Reynolds, and Harold Lichtenstein 173

EFFECTS OF LIGHT ON THE FORMATION OF CAROTENOIDS IN TOMATO FRUITS. J. P. McCollum 182

RAW STARCH DIGESTION: A COMPARISON OF THE RAW STARCH DIGESTING CAPABILITIES OF THE AMYLASE SYSTEMS FROM FOUR ALPHA-AMYLASE SOURCES. R. M. Sandstedt and R. L. Gates 190

DENATURATION OF MYOSIN DURING DEFROSTING OF FROZEN FISH. Olavi E. Nikkilä and Reino R. Linko 200

ERRORS OF THE SECOND KIND IN ORGANOLEPTIC DIFFERENCE TESTING. G. A. Baker, M. A. Amerine, and E. B. Roessler 206

THE LEAD, ZINC AND COPPER CONTENT OF FOODS. Doris Larkin, Margaret Page, J. C. Bartlet, and Ross A. Chapman 211

AMINO ACID COMPOSITION OF ORGAN MEATS. B. S. Schweigert, Barbara A. Bennett, and Barbara T. Guthneck 219

NUTRITIVE VALUE OF MILK PROTEINS. II. STABILITY DURING STERILIZATION OF EVAPORATED MILK AS DETERMINED BY THE RAT GROWTH METHOD. A. Z. Hodson. 224

COMPOSITION OF CERTAIN SPECIES OF FRESH-WATER FISH. I. INTRODUCTION: THE DETERMINATION OF THE VARIATION OF COMPOSITION OF FISH. M. E. Stansby . 231

BIURET METHOD FOR SOLUBLE WHEY PROTEINS IN NONFAT DRY MILK SOLIDS. Harold Salwin 235

ROPY MILK. I. THE BIOLOGY OF ROPE PRODUCTION. Daniel L. Jones 246

ROPY MILK. II. THE INFLUENCE OF PHYSICOCHEMICAL ENVIRONMENT ON THE FORMATION OF ROPY SUBSTANCE. Daniel L. Jones 250

ROPY MILK. III. CHEMICAL STUDIES OF ROPY SUBSTANCE. Daniel L. Jones . . . 254

COMPARISON OF THE NUTRITIVE VALUES OF MONO-, DI-, AND TRIGLYCERIDES BY A MODIFIED PAIR-FEEDING TECHNIQUE. Robert S. Harris and Henry Sherman . . 257

SUBSCRIPTION RATES

To members of the Institute of Food Technologists, $5.50 per volume.

Non-members: Domestic, $6.50 per volume; foreign, $7.00 per volume.

Subscriptions and renewals are entered to begin with the first issue of the current volume.

Single copies of current volume: Domestic, $1.00; foreign, $1.15.

CORRESPONDENCE

Editorial: MARTIN S. PETERSON, Editor-in-Chief, 11606 South Bell Ave., Chicago 43, Ill.

Non-Member Subscriptions, Back Volumes, Single Copies, and Reprints: FOOD RESEARCH, 119-123 W. Park Ave., Champaign, Ill.

Members Subscriptions: THE INSTITUTE OF FOOD TECHNOLOGISTS, 176 W. Adams St., Chicago 3, Ill.

Advertising: Charles S. Lawrence, Business Manager, 176 W. Adams St., Chicago, Ill.

CLAIMS

Claims for copies lost in the mails must be received within 30 days (90 days foreign) of the date of issue. Notice of change of address must be received two weeks before date of issue.

NOTE

The Institute of Food Technologists assumes no responsibility for statements in articles appearing in FOOD RESEARCH. The opinions expressed do not necessarily reflect policies of the Institute of Food Technologists.

Manuscripts for FOOD RESEARCH should conform to the style used in the journal and be submitted in duplicate to the Editor-in-Chief. Authors of papers intended for FOOD RESEARCH should request from the Editorial Office a copy of the booklet "Information and suggestions for authors." FOOD RESEARCH reserves the privilege of editing manuscripts to make them conform with the adopted style of the journal or returning them to the author for revision. Changes made by the Editorial Office may be reviewed by authors when they examine proof.

Copyright, 1954
By the Institute of Food Technologists
Printed in U. S. A.

Here's the Answer to . . .

YOUR CONTROL PROBLEMS

For quick, dependably accurate readings in quality control, in "on-the-spot" production line or field checks, or in research—choose from the complete Bausch & Lomb refractometer line, industry's standard.

Let us show you the B&L instrument that can most efficiently solve *your* specific problem . . . save you time, money and materials. No obligation, of course.

Write for complete information and a demonstration to Bausch & Lomb Optical Co., St. Paul St., Rochester 2, N. Y.

Bausch & Lomb **REFRACTOMETERS**

METHODS OF GRADING PECTIN IN RELATION TO THE MOLECULAR WEIGHT (INTRINSIC VISCOSITY) OF PECTIN[a]

FOVL E. CHRISTENSEN
The Copenhagen Pectin Factory, Copenhagen, Denmark

(Manuscript received June 29, 1953)

The literature published during the last 20 years on the grading of pectin contains many different methods. From among these, however, it has been agreed to hold in disfavor those methods which are not based on the principle of cooking test jellies with sugar and acid. The methods remaining after this selection can be divided into two large groups, classification being dependent on the property of the test jellies considered to be the most important.

Group I. In Group I methods of measurement, the elastic limits of the jellies (breaking strength) are exceeded and the jellies ruptured.

a. Sucharipa's jelly disc. method *(21)*
b. Fellers-Clague's penetrometer method *(8)*
c. Lüers-Lochmüller's "Pektinometer" *(12)*
d. Delaware Jelly Strength Tester by Tarr-Baker *(1, 22)* and several modifications of same.

Group II. In these methods the instrument measures the deformation of the jellies *(sag)* without exceeding the elastic limits.

a. Bloom's gelometer *(5)*
b. Cox and Highby's *sag* method *(7)*, and several modifications
c. B. A. R. Jelly Tester *(6)*
d. Säverborn's cylindrical torsion method *(18)*.

The methods under Group I differ essentially in the design of the measuring instrument used in the determination of the breaking strength of the test jellies. This also applies to the methods under Group II.

Our experience is that the cooking formula as well as the cooking procedure in both groups have an influence on obtaining reproducible results.

The definition of the term "grade" in connection with pectin differs also, but it seems as if the American definition is now so well accepted in most pectin-producing countries, e.g., in the U.S.A., Germany, Switzerland, France, England, Scandinavia, Belgium, etc. that there is no longer any controversy concerning this point.

The texture of the test jelly has been much discussed in recent years, but this important question is still not settled.

It will certainly not be difficult to obtain agreement on the cooking formula and procedure, especially in view of the fact that the most important use of pectin is in jam, marmalade and jelly manufacture where the examination of the pectin must be as close to the practical use of pectin as possible. As to analytic methods of testing pectin, we suppose

[a] Presented at the Pectin Symposium of the New York Preservers Association, New York, February 5, 1953.

the problems are: (a) whether the method under Group I or II should be used, and (b) whether the chemical characteristics of the pectin have any influence on the texture of the jelly.

By proper choice of cooking procedures and by proper fixing of the strength of the standard jellies it will in our opinion be possible to modify the methods under Group I or II so that the results within each group will give identical grade values for the pectin analyzed. This is not to say that correspondence between Groups I and II is obtained.

Comparing the results achieved by a *breaking strength* method with those of a *sag* method we find that it is impossible to get grade values which coincide for all pectins. This is perhaps the reason why in many countries pectin committees are set up to work out a method of grade determination or to decide which of the existing methods is to be preferred (e.g., which method corresponds best with practice).

EXPERIMENTAL

Determination of viscosity. The viscosity of the pectin solutions was determined by an Ostwald capillary viscosimeter at 20°C. The flow time for water was 122.82 seconds.

The calculation of the relative viscosity was made according to the following equation, taking into account the correction of kinetic energy according to Hagenbach *(16)*.

$$\eta_r = \frac{\left(t_s - \frac{K}{t_s}\right)\rho_s}{\left(t_o - \frac{K}{t_o}\right)\rho_o} \qquad (1)$$

where

t_s is the flow time of the pectin solution;
t_o is the flow time for the 1% sodium-hexametaphosphate;
ρ_s is the specific gravity of the pectin solution;
ρ_o is the specific gravity of the 1% sodium-hexametaphosphate;

K is a constant equal to the following equation:

$$K = \frac{Q\,t_w^2}{Q + \frac{\eta_w\, 8\pi\, L\, t_w}{1.12\,\rho_w}}$$

t_w is the flow time for water at 20°C.;
ρ_w is the specific gravity of water at 20°C.;
η_w is the viscosity of water at 20°C.

The procedure for measuring the viscosity of the pectin solution has been to use sodium-hexametaphosphate as supporting and complexing electrolyte as in the investigations of Lotzkar *et al. (11)* and Owens *et al. (15)*.

Reagent. Ten g. of sodium-hexametaphosphate are dissolved in 900 ml. of distilled water and pH is reduced to 4.5 by adding 1 n. hydrochloric acid and diluting with water to a total volume of 1,000 ml.

0.500 g. pectin (depending on the grade value) are weighed out, poured into a 150-ml. beaker, and dissolved in 90 ml. 1% sodium-hexametaphosphate under constant stirring. After complete solution the total volume is brought up to 100 ml. by adding the phosphate solution. The solution is filtered through a dry ash-free filter paper in order to remove possible undissolved substances.

Five ml. of the solution are pipetted off in the viscosimeter; this is placed in the carefully controlled constant temperature bath at 20°C. After 15 minutes the flow time is measured 5 times.

Fifty ml. of this solution is now diluted with 50 ml. phosphate solution, and the flow time for this solution is measured and so on. The viscosity of the pectin solution

is always measured at 4 different concentrations of pectin, i.e., 0.5, 0.25, 0.125 and 0.0625 g. per 100 ml.

The average of the flow times for each dilution is found and their relative viscosity is calculated, using formula 1.

The following quantities are computed:

$$\eta_{sp} = \eta_r - 1; \frac{\eta_{sp}}{c} \text{ and } \frac{(\eta_r^{1/p} - 1)p}{c} = [\eta]$$

The last formula is a conversion of Baker's equation *(2)*

$$\eta_r = (1 + ac)^p$$

which for small values of c gives:

$$\eta_r = 1 + pac; \eta_{sp} = \eta_r - 1 = pac; \quad [\eta] = pa = \frac{(n_r^{1/p} - 1)p}{c}$$

$$[\eta] = \operatorname{Lim}_{c \to o} \frac{\eta_{sp}}{c}$$

according to definition by G. V. Schulz *(19)*.

In the calculation we have used $p = 6$, this value of p giving the smallest differences in $[\eta]$ values. In Table 1 we have given the result of such a calculation.

TABLE 1

c g./100 ml.	Flow time min.	η_r	η_{sp}	$\frac{\eta_{sp}}{c}$	$\frac{(\eta_r^{1/6}-1)6}{c}$
0.40	395.46	3.110	2.110	5.275	3.123
0.20	225.96	1.775	0.775	3.874	3.009
0.10	171.50	1.345	0.345	3.451	3.042
0.05	148.78	1.166	0.166	3.312	3.108

CALCULATION OF THE MOLECULAR WEIGHT

The molecular weight is calculated by using Staudinger's equation *(20)*

$$[\eta] = \operatorname{Lim}_{c \to o} \frac{\eta_{sp}}{c} = K M$$

where K is a constant and M is the molecular weight.

The intrinsic viscosity $[\eta]$ is found by extrapolating. This is done in such a way that the values

$$\left(\frac{\eta_{sp}}{c}, c\right) \text{ and } \left(\frac{(\eta_r^{1/6} = 1)6}{c}, c\right)$$

are plotted on ordinary graph paper as seen in Figure 1, and a smooth curve drawn through the points. The reason for drawing 2 curves is that the intrinsic viscosity can be determined with somewhat greater accuracy.

From Figure 1 the intrinsic viscosity is found to be 3.24.

The value of K is not yet known with reasonable accuracy—nor is the influence of the molecular weight and the methoxyl content of the pectin on same thoroughly investigated. B. Vollmert *(23)* has shown that the value of K for high methoxyl pectins is nearly constant.

Although the influence of the molecular weight on the value of K is not yet investigated, it is shown that the influence of M on the value of K for other linear macromolecules is only small in the region $30{,}000 < M < 200{,}000$. In this investigation we have chosen the value 4.7×10^{-5}, but a possible change of this value has no influence on the conclusions of this investigation. For the pectin in question we calculate

$$M = \frac{3.24}{4.7 \cdot 10^{-5}} = 69{,}000$$

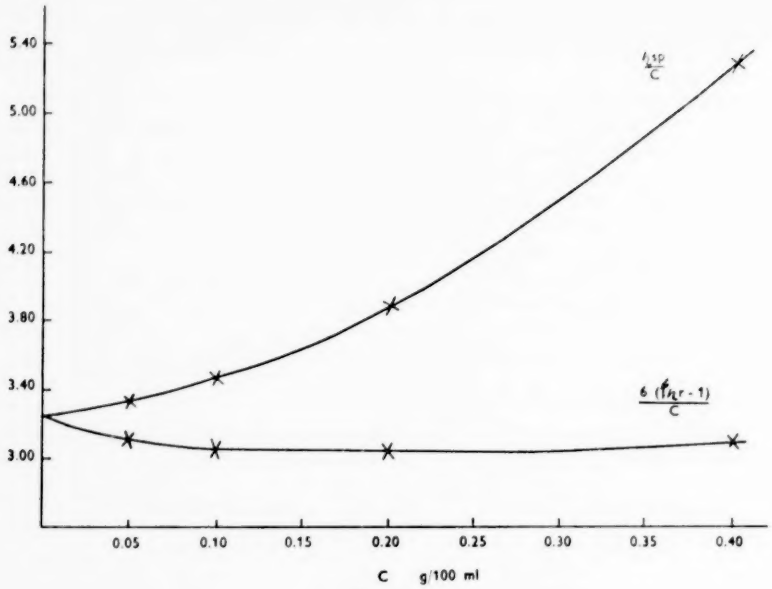


Figure 1

The calculated molecular weight of the pectin is then converted to 100% polyuronide content.

Determination of the polyuronide content of pectin. As shown by Lefévre-Tollens *(10)* uronic-acids are completely decarboxylated by boiling with strong hydrochloric acids. The liberated Co_2 is absorbed in $Ba(OH)_2$-solution, and the amount of CO_2 evolved can be determined in this way. This method has been modified several times. The method used by us is worked out by McCready, Swenson, and Maclay *(13)*. But we have changed the apparatus a little in order to increase the accuracy. This determination is rapid and accurate, taking only 2 hours. The calculation of the percent pectin in the sample is done according to the following equation:

$$\frac{8{,}80 \cdot 190 \cdot \text{Aeq} \cdot \text{a} \cdot \text{n}}{176\ (\text{Aeq} + 14) \cdot \text{b}} = \% \text{ pectin}$$

where

Aeq is the acid-equivalent weight of the pectin;
a is the amount of $Ba(OH)_2$ in ml. used.
n is the normality of the $Ba(OH)_2$-solution;
b is the amount of the pectin sample in g.

We have not determined the acid-equivalent weight of the pectin samples, but we have chosen the value of 550 for the sample of the slow set types and the value 750 for the rapid set types.

Jelly grade determination of the pectins investigated. a) *The Sag Method.* For this determination we have used the method developed by the British Food Manufacturing Research Association *(17)*. This method is a modification of the method developed by Cox and Highby, California Fruit Growers Exchange *(7)*. The instrument used for measuring the jelly strength is the "ridgelimeter" (California Fruit Growers Exchange) and the jelly glasses used have precisely the same dimensions. The cooking of the test jellies is done exactly as prescribed in *Analyst* and differs from that of Cox and Highby, as the jelly is cooked in the presence of buffer solution, and has a soluble solids content

of 70.5% as opposed to 65%. The grade values found by this method are, according to *Analyst*, exactly the same as if the original Cox and Highby method were used. This we also nearly achieved, but according to our experience it seems as if the grade values found according to the English method are about 5-10% higher for the rapid set types of pectin than the values found by the Cox and Highby method. The reason why we have used the English method and not the Cox and Highby method, is that, with G. L. Baker *(3)*, we consider it important that the test jelly be cooked in a way similar to practical cooking, i.e. the buffer must be present under the cooking, and the pH in cooked jelly must be about 3.1 as in commercial practice and not 2.0-2.2 as in the method of Cox and Highby. (Our experience is that it is very difficult to get the acid evenly distributed in the jelly in the "acid in glass" method, especially when cooking jellies with rapid set pectins).

b) *The Breaking Strength Method.* The cooking of the test jellies is done in the following way and after this recipe:

1.3 g. 100° pectin
34.0 ml. buffer solution
121.7 g. sugar
50.0 ml. distilled water.

The pectin is weighed out on an analytical balance and mixed dry with 10 g. of the sugar in a 400-ml. tared beaker. Buffer solution and water are added, and the whole heated to a boil and boiled vigorously for ½ to 1 minute.

When the pectin is dissolved, the remaining quantity of sugar is added in 3 or 4 portions, heating constantly. After that the whole is boiled down to a total weight of 200 g. and the cooked jelly is poured into 5 jelly rings (inside diameter 35 mm. and height 27 mm.). Each jelly ring is placed in advance on a glass plate. The ring must be filled up and allowed to stand completely still for 3 hours at 20°C. ± 2°, whereupon the jelly strength is measured as described below.

The instrument used to measure the breaking strength of the test jellies is similar to the "Delaware Jelly Tester" with the modification that we used water instead of carbontetrachloride, and the dimensions of the piston are different. The latter is not important, if the breaking strength is given as g. per cm^2. Before measuring the test jellies these are turned, and then the glass plates and the rings are removed. The soluble solids content of the jellies must be 65.0 ± 0.5 and pH 3.00 ± 0.05.

The buffer solution has the following composition:

16.0 g. calcium-citrate $Ca_3(C_6H_5O_7)_2$, $4H_2O$
152.0 g. citric acid $C_6H_8O_7$, H_2O
220.0 g. sugar
38.4 g. potassium citrate $K_3C_6H_5O_7$, H_2O
distilled water to 2,000 ml.

CALCULATION OF THE GRADE VALUE

The grade value is calculated from the following equation:

$$2.4 \times \log (\text{grade}) + 2.4 \times \log (\text{g. pectin}) = \log (\text{mm. water pressure}) + 2.2053.$$

This equation in its logarithmic form is well known from Tarr-Baker and others, e.g., Bender *(4)*, and the constants are determined by using this equation on different pectins from all over the world. The standard jelly has, according to this equation, a breaking strength of 40 g. per cm^2 corresponding to 738 mm. water pressure on our instrument. The grade values computed according to the equation above closely correspond with those found with the original "Delaware Tester," and give results comparable with those found by the common finger test.

Normally, test jellies are cooked with 3 different amounts of pectin, and the 3 so computed grade values lie normally within ± 2% from the average. According to the above methods the results of several analyses of different pectins are given in Table 2. In this table we have the results of analyses of both slow set and rapid set types of pectin extracted from different raw materials, e.g., lemon, grape, orange and apple.

TABLE 2

Effect of molecular weight of pectin on the "grade-value" of same

No.	Pectin	Origin	Setting time	Claimed "grade"	Percent 100% polyuronide	Intrin. visc. (η)	Intrin. visc. calc. for 100% polyuronide	Mol. weight $\cdot 10^3$	Breaking strength "grade"	Breaking strength "grade" calc. for 100% polyuronide	"Sag" grade	"Sag" grade calc. for 100% polyuronide	(Grade) "Br." : (Grade) "Sag"
I	II	III	IV	V	VI	VII	VIII	IX	X	XI	XII	XIII	XIV
1	Laboratory Citrus		Rapid		71.5	7.20	10.1	215	376	526	255	357	1.38
2	Commercial Apple	Denmark	Medium	180	38.3	3.68	9.62	205	184	482	137	357	1.35
3	Commercial Apple	Germany	Slow	200	44.8	4.21	9.40	200	207	464	150	335	1.38
4	Commercial Apple	Denmark	Slow	180	38.5	3.58	9.30	198	181	471	134	348	1.36
5	Commercial Citrus	U.S.A.	Slow	150	50.0	4.08	8.16	174	182	364	163	326	1.11
6	Commercial Citrus	U.S.A.	Rapid	150	48.8	3.86	7.91	169	189	387	147	302	1.28
7	Commercial Citrus	U.S.A.	Slow	150	47.0	3.53	7.90	168	176	375	146	312	1.20
8	Commercial Citrus	Denmark	Slow	200	54.2	4.24	7.80	166	202	372	168	308	1.21
9	Commercial Citrus	Denmark	Rapid	200	54.3	4.18	7.70	164	205	378	172	316	1.20
10	Commercial Apple	Switz. 1	Medium	250	71.2	5.40	7.57	161	236	332	195	273	1.22
11	Commercial Citrus	U.S.A.	Rapid	150	48.2	3.48	7.23	154	160	332	143	297	1.12
12	Commercial Citrus	U.S.A.	Rapid	150	51.8	3.37	6.52	139	153	296	141	272	1.09
13	Commercial Citrus	U.S.A.	Slow	150	48.9	3.16	6.48	138	140	287	139	285	1.01
14	Commercial Apple	U.S.A.	Rapid	150	55.6	3.52	6.35	135	150	270	142	256	1.05
15	Commercial Citrus	U.S.A.	Rapid	150	50.5	3.13	6.20	132	121	240	126	250	0.96
16	Commercial Apple	Germany	Rapid	100	27.5	1.67	6.07	129	66	239	69	253	0.95
17	Commercial Citrus	U.S.A.	Slow	150	63.6	3.75	5.91	126	162	255	170	267	0.96
18	Commercial Citrus	Italy	Rapid	100	41.6	2.40	5.76	122	90	218	100	242	0.90
19	Laboratory Orange		Rapid		75.2	3.72	4.95	105	148	197	175	232	0.85
20	Laboratory Grapefruit		Rapid		84.0	3.20	3.82	81	99	118	165	196	0.60
21	Heat treated App.		Rapid		64.0	1.41	2.20	47	45	70	72	113	0.62
22	Enzyme treated App.		Rapid		42.5	0.870	2.05	44	20	47	51	120	0.39

All the pectins mentioned are commercial pectins except 1, 19, 20, 21, and 22.
Pectin No. 21 is made by heating dried apple pectin in a closed container 2½ months in a thermostat at 50°C.
Pectin No. 22 is made by treating a pectin solution with a commercial pectinase enzyme and precipitating the pectin by addition of ethyl alcohol, drying and grinding.

RESULTS AND DISCUSSION

Figure 2 is on ordinary graph paper giving the relation between the molecular weight of the pure pectin and the *sag* grade value also calculated for the pure pectin, and in the same way the relation between the molecular weight and the *breaking strength* grade values. The curves are not drawn through the point (0.0), as in the region near this point the test jellies must be cooked with such considerable amounts of pectin that they are not comparable with normal sugar jellies.

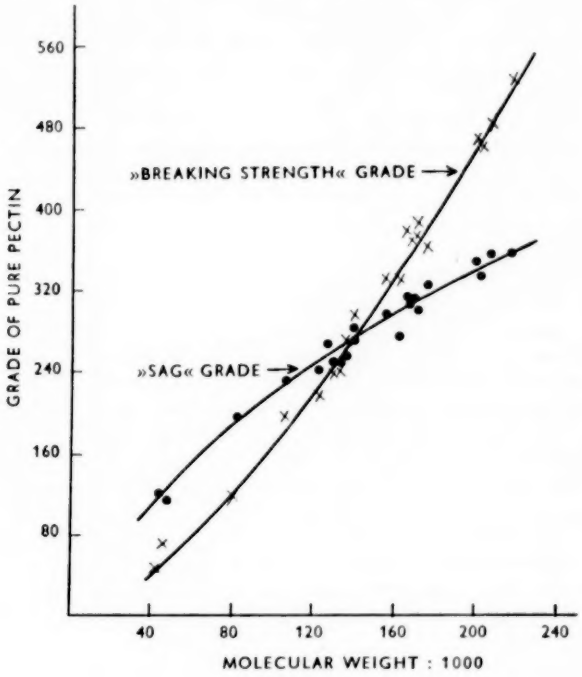


Figure 2

From Table 2 it is seen that the results with pectin No. 21 do not fit as closely in the system as the others. The reason is without doubt that this pectin after the degrading treatment contains some pectin of very low molecular weight. All the others are partly fractionated by precipitating with alcohol or treated in precipitated form with alcohol, thereby removing the molecules with the lowest degree of polymerization.

Taking into consideration the uncertainty there is in the determination of the intrinsic viscosity and the degree of purity of the pectins, it is seen that the points (M, grade) fit closely to the curves drawn in Figure 2.

The explanation as to why the American pectin literature does not mention the pronounced difference between the grade values determined by the 2 test methods can perhaps be that the American pectins used by

the investigators probably have had a molecular weight of about 135,000 [the only exception is H. S. Owens *(14)*]. On the basis of the samples of commercial pectins from U.S.A. it seems as if the molecular weight of these in the last few years is increasing (the pectins in Table 2, Nos. 12 and 17, are from 1949; Nos. 5 and 6, from 1952). According to this theory it is easily understood that an attempt has been made to find other reasons for the relatively small differences ascertained according to the *breaking strength* and the *sag* method. Consequently, it has been difficult to decide which method gives results corresponding to the practical use of the pectin in marmalade, jam, and jelly manufacture.

As shown in Table 2 only pectins having a molecular weight of about 135,000 — whether manufactured from citrus or apple waste — gave the same result by the use of the *sag* and the *breaking strength* methods; the grade value of such a pectin based on the pure pectin will be 280°. Further the table shows that pectins with a lower molecular weight than 135,000 give a higher result by the *sag* method than by the *breaking strength* method, and, vice versa, pectins with a higher molecular weight than 135,000 give a higher result by the *breaking strength* method than by the *sag* method.

The value of this finding can, in our opinion, only be estimated by comparing the results with the jellifying ability of the laboratory-tested pectins in practice in commercial batches.

To simplify this work we have, however, chosen an easier way; namely, to test the pectins according to finger test. The results of this test are good and reproducible, when the analysts have adequate experience in estimating the jellies. The finger test chosen by us is prescribed by U. S. Department of Agriculture *(9)*. This method was used in pectin purchases during the second world war. The test jellies are subjected to the usual finger-testing and judged as to the texture, resilience, and consistency. Under certain conditions this method gives results most closely corresponding to practice. The only disadvantage in using the method is that we are forced to cook a standard jelly from a standard pectin in order to get exact grade values—the keeping qualities of the standard pectin are unknown, and the characteristics of the standard jelly are not fixed.

As mentioned above, there is correspondence between the *sag* and the *breaking strength* measurings of pectins with a molecular weight of 135,000. The experiments with finger test will show whether the grade value according to the *sag* or the *breaking strength* method is correct. Among the several tests performed with the selected pectins, we have chosen the following, mentioned in Table 3.

As standard pectin we used No. 13, a citrus pectin originating in the U.S.A.

According to the opinion of all analysts the jellies containing "500 Breaking Strength Jelly Units" corresponded closely to the standard jelly, whereas the jellies containing "500 Sag Jelly Units" were considerably firmer for pectin No. 3 and considerably weaker for pectin No. 20. On the other hand it must also be mentioned that there is a great difference between jellies cooked with the same number of *breaking strength* jelly units, if the molecular weights of the pectins are far apart even

though the finger test shows the same strength. Jellies cooked with pectins of a high molecular weight have a smoother consistency and a greater elasticity than jellies made with pectins of lower molecular weight.

TABLE 3

Test	Pectin No.	Sag grade	Breaking strength grade	Pectin used—grams	Sag jelly units	Breaking strength jelly units
1	13	139	140	3.58	500	500
2	3	150	207	3.33	500	690
3	3	150	207	2.42	363	500
4	20	165	99	3.03	500	299
5	20	165	99	5.05	833	500

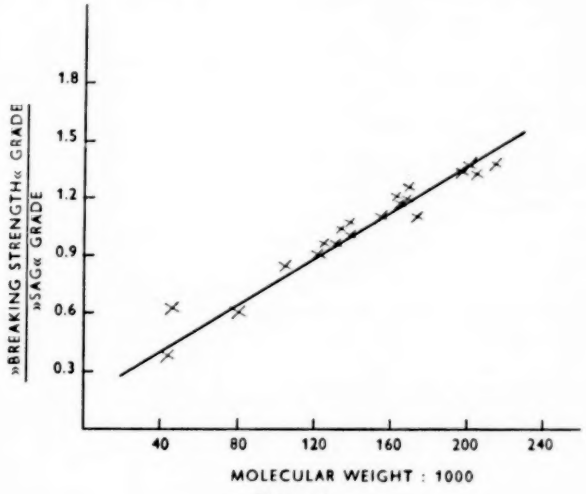


Figure 3

SUMMARY

The relationship between the molecular weight of pectin and "grade value" has been determined. It is shown that the proportion between "*sag* grade value" and "*breaking strength* grade value" of pectin is not constant, but depends on the molecular weight of the pectin. This means that it will be impossible to alter the existing methods of grade determination so that the *sag* method will give grade values which coincide with the values determined by the *breaking strength* method.

LITERATURE CITED

1. Baker, G. L. *Ind. Eng. Chem.*, **18**, 89 (1926).
2. Baker, G. L. *J. Chem. Soc.* (London) 103, 1653 (1913).
3. Baker, G. L., and Woodmansee, C. W. Delaware Agr. Expt. Sta. *Bull. 272* (1948).
4. Bender, W. A. *Ind. Eng. Chem. Anal.*, **21**, 408 (1949).
5. Bloom, O. T. U. S. Pat. 1,540,979 (1925).

6. CAMPBELL, L. E. *J. Soc. Chem. Ind.*, **57**, 413 (1938).
7. COX, R. E., AND HIGHBY, R. H. *Food Inds.*, **16**, 441 (1944).
8. FELLERS, C. R., AND CLAGUE, J. A. *Ind. Eng. Chem., Anal. Ed.*, **4**, 106 (1932).
9. KERTESZ, Z. I. *The Pectic Substances*, New York (1951).
10. LEFÉVRE, K. U., AND TOLLENS, B. *Ber.* **40**, 4513 (1907).
11. LOTZKAR, H., SCHULTZ, T. H., OWENS, H. S., AND MACLAY, W. D. *J. Phys. Chem.*, **50**, 200 (1946).
12. LÜERS, H., AND LOCHMÜLLER, K. *Kolloid-Zeitschrift*, **42**, 154 (1927).
13. MCCREADY, R. M., SWENSON, N. A., AND MACLAY, W. D. *Ind. Eng. Chem.*, **18**, 290 (1946).
14. OWENS, H. S., LOTZKAR, H., MERVILL, R. C., AND PETERSON, M. *J. Am. Chem. Soc.*, **66**, 1178 (1944).
15. OWENS, H. S., PORTER, O., AND MACLAY, W. D. *Food Ind.*, **19**, 606 (1947).
16. PHILIPPOFF, W. Viscosität der Kolloide, Dresden und Leipzig (1942).
17. Report of the Pectin Sub-Committee of the Jam Panel, British Manufacturing Industries Research Association. *Analyst* **76**, 536 (1951).
18. SÄVERBORN. A contribution to the knowledge of the acid polyceronides (1945). Dissertation. Uppsala (1945).
19. SCHULZ, G. V. *J. prakt. Chem.*, **158**, 130 (1941).
20. STAUDINGER, H. Die hochmolekularen Verbindungen, Berlin (1932).
21. SUCHARIPA, R. *J. Assoc. Offic. Agr. Chemists*, **7**, 57 (1923).
22. TARR, L. W. Delaware Agr. Expt. Sta. *Bull. 142* (1926).
23. VOLLMERT, B. *Die Makromolekulare Chemie. Ed.*, **5**, Heft 2, 128 (1950).

SIGNIFICANCE OF VARIATIONS IN OBSERVED SLOPES OF THERMAL DEATH TIME CURVES FOR PUTREFACTIVE ANAEROBES

ARTHUR M. KAPLAN, HOWARD REYNOLDS, AND HAROLD LICHTENSTEIN

Bureau of Human Nutrition and Home Economics, Agricultural Research Administration, United States Department of Agriculture, Washington, D. C.

(Manuscript received July 25, 1953)

Information on the thermal resistance characteristics of specific spoilage organisms is needed to estimate the lethalities of heat treatments applied in the pasteurization or sterilization of foods. Data now in the literature provide substantial basis for the assumption that, for a given organism under specified conditions, thermal death time is an exponential function of temperature. Thus, the resistance characteristics of a spoilage organism are designated by a thermal death time curve which, for practical purposes, is a straight line on semilogarithmic paper defined by a slope or z value and a point or F value *(1)*. These data are also sufficient to demonstrate that the F value for a given organism depends upon the nature of the medium in which its determination is made *(5, 6)*.

Slope or z value of the thermal death time curve also appears to be influenced by the medium in which it is determined but relevant data are less conclusive. Thus, reported heat resistance data for spores of Cameron's Putrefactive Anaerobe 3679, a typical low-acid canned food spoilage organism, can be interpreted as evidence that z values of its thermal death time curves vary when determined in different food substrates *(6)*. However, it is also notable that replicated determinations in a single food substrate have yielded z values exhibiting a range of variation comparable to that observed from determinations in several different substrates. For example, Townsend, Esty, and Baselt *(14)* using asparagus, peas, spinach, and milk as substrates, observed composite z values for P. A. 3679 spores varying from 16.9 to 21.1. At the same time individual determinations in asparagus yielded z values ranging from 16.6 to 30.0.

In a previous paper, Reynolds, Kaplan, Spencer, and Lichtenstein *(6)* reported data on the characteristics of thermal death time curves for P. A. 3679 spores heated in 13 different vegetable substrates. The purpose of this report is to present additional thermal death time data on P. A. 3679 and *Clostridium botulinum* spores and to determine whether the variations in z values that were observed when spores were heated in a number of different substrates, represent real interactions between substrate and relative spore resistance or whether they can be attributed to uncontrolled variables in the experimental methodology used.

METHODS

The methods, media, techniques, and nomenclature used in this study were those previously described *(6)*. Spore suspensions of *Clostridium botulinum* 213 B were prepared

in the same manner as suspensions of P. A. 3679 and were stored in the refrigerator without any prior heat treatment.

Ground round steak and pork loin, trimmed of fat and bone, were used as the meat substrates. Fill-in weights for the thermal death time cans were 10 g. of meat and 4 ml. of spore suspension. Covariance and regression analyses were carried out according to Snedecor *(9)*.

RESULTS

In order to study statistically the variation in z values obtained by heating P. A. 3679 in various substrates, it was necessary to determine the coefficient of regression of log D value on temperature for each individual thermal death time curve. The z values, calculated as the reciprocals of the regression coefficients, are presented in

TABLE 1

z values calculated by regression analysis from log D values for 10,000 P. A. 3679 spores heated in food substrates

Product	With medium	Without medium
Beef	21.2	.....
Pork	18.3	.....
Asparagus	18.9	13.4
Beans, lima	19.3	11.8
Beans, snap	19.0	14.2
Beets	19.1	18.9
Carrots	18.5	18.6
Corn	19.8	20.0
Mushrooms	16.7	22.0
Okra	16.8	13.5
Peas	17.8	15.9
Potatoes, sweet	15.5	19.1
Pumpkin	19.2	17.0
Spinach	18.2	19.1
Squash	22.1	20.7
Average*	18.7	17.8
Standard deviation	1.67	3.20
Average, excluding lima beans and okra*	.....	18.3
Standard deviation	.....	2.67

*Obtained from covariance analysis.

Table 1 for P. A. 3679 heated in 15 food substrates and incubated after supplementation of the food with buffered peptone medium and in 13 food substrates incubated without added medium. D values used in these determinations were calculated according to Schmidt's method *(7)* and are those previously presented by Reynolds, Kaplan, Spencer, and Lichtenstein *(6)* augmented with additional data (Table 2) for beef, pork, peas, lima beans, corn, and mushrooms incubated with added medium, and mushrooms incubated without added medium. The value, log 0.5, used in Schmidt's formula makes the assumption that at the time corresponding to LD_{50} there were theoretically 0.5 spores per container remaining viable on the average. Schmidt *(8)* has noted, however, that log 0.692, calculated as described by Halvorson and Ziegler *(4)* is a more accurate approximation of the expected number of survivors. Recalculations of a random sampling of the data using the latter value yielded D values approximately 4% higher than those previously reported *(6)* and those listed in Table 2. The z values of thermal death time curves drawn through the new D values or fitted to the new data by regression methods, however, did not differ from those obtained with the previously calculated D values. Thermal death time curves drawn freehand through observed D values had z values in very close agreement with those fitted to the data by regression methods.

Covariance analyses of the regression coefficients of the thermal death time curves of P. A. 3679 spores heated in 15 food substrates and incubated with added medium

TABLE 2

Calculated times for 90 percent destruction of P. A. 3679 spores heated in some food substrates and incubated with added medium

Product	D value for P. A. 3679 spores at				
	250°F.	245°F.	240°F.	235°F.	230°F.
	(min.)	*(min.)*	*(min.)*	*(min.)*	*(min.)*
Beef	2.23	3.74	5.28	10.35	20.19
Pork	2.14	4.56	9.63	15.35	27.21
Beans, lima	2.56	4.74	7.74	14.77	28.60
Corn	2.00	4.40	6.58	11.74	22.56
Mushrooms[b]	1.74	2.91	4.98	.....	.....
Mushrooms	1.98	3.54	6.56	15.35	30.70
Peas	2.23	3.77	9.07	16.23	26.98

[b] Incubated without added medium.

indicated that the over-all slope, calculated as z = 18.7, could be taken as representative of each of the individual slopes since the latter did not vary significantly among themselves at the 5% level (Table 3). Individual curves had calculated z values ranging from 15.5 to 22.1.

When z values were calculated from survival data for spores heated in 13 food substrates and incubated without addition of medium, the over-all slope (z = 17.8), could not be substituted for all individual slopes since the latter varied significantly among themselves at the 1% level. Individual slopes had calculated z values ranging from 11.8 to 22.0. When data for lima beans (calculated z = 11.8) and okra (calculated z = 13.5) were removed from the calculations, the over-all slope for the 11 remaining substrates (z = 18.3) could be tentatively accepted as describing each slope since the 11 remaining z values did not vary significantly among themselves at the 5% level. Slopes for this group ranged from 13.4 to 22.0, a range which encompasses the previously discarded z value of 13.5 for okra. This apparent discrepancy is attributable to an unusual sampling variation and exceptionally high standard error for the asparagus regression from which the included z value of 13.4 was derived. This variation was so large, in fact, that the data provide no basis for concluding that the true z value for asparagus is lower than the average of 18.3. On the other hand, the excluded z value of 13.5 for okra was provided by regression data with a low standard error which statistically isolated that slope from the group mean slope of 18.3.

The analyses (Table 3) also show that determination of survival-destruction times by incubation of processed thermal death time cans, either with or without added medium, had no significant effect on the calculated average slope values of thermal death time curves.

F values for the thermal death time curves of 10,000 spores of P. A. 3679 heated in beef, pork, corn, lima beans, peas, and mushrooms, incubated with medium supplementation and beef, pork, and mushrooms incubated without medium are listed in Table 4. For comparative purposes, previously reported *(6)* F values for P. A. 3679 spores heated in corn, lima beans, and peas and incubated without added medium are included in Table 4. F values for P. A. 3679 heated in beef and pork and incubated without medium supplementation are based on survival-destruction data at 250°F. (121.1°C.). Higher F values were obtained in all instances when the heated substrates were incubated with added medium.

Thermal death time data were obtained for 25,000,000 spores of *Cl. botulinum* 213-B heated in snap beans, beets, carrots, corn, and spinach, and 200,000 spores heated in Sorenson's M/15 neutral phosphate buffer. Buffered peptone medium was added to the food substrates before incubation; the phosphate suspensions were subcultured in tryptone yeast extract agar. D values calculated by the methods of Stumbo *(11)* and Schmidt *(7)* are presented in Table 5 while F and z values are presented in Table 6. The z values plotted from survival-destruction end points, from D values calculated by the methods of Stumbo and Schmidt and from regression analysis agreed well one with another.

TABLE 3

Summary of tests of significance of differences among regression coefficients of log D values on temperature for P. A. 3679 spores heated in various low-acid foods

Differences tested and source of variation	Degrees of freedom	Sum of squares	Mean square[c] (variance)	Variance ratio		
				Observed	5-percent level	1-percent level
Test of differences among individual (substrate) regressions according to method of incubation:						
With medium, all substrates studied:						
Deviations from pooled average regression within substrate	47	.147724				
Deviations from individual regressions within substrate	33	.096102	.002912			
Remainder, differences among regression coefficients for individual substrates	14	.051622	.003687	1.27	2.01	2.68
Without medium, all substrates studied:						
Deviations from pooled average regression within substrate	33	.208469				
Deviations from individual regressions within substrate	21	.071430	.003401			
Remainder, differences among regression coefficients for individual substrates	12	.137039	.011420[d]	3.36	2.25	3.17
Without medium, excluding lima beans and okra:						
Deviations from pooled average regression within substrate	29	.145741				
Deviations from individual regressions within substrate	19	.070198	.003695			
Remainder, differences among regression coefficients for individual substrates	10	.075543	.007554	2.04	2.38	3.43
Test of differences between average regressions for each method of incubation:						
With medium, all substrates studied versus without medium, all substrates studied:						
Deviations from pooled average regression for both methods of incubation	81	.363032				
Deviations from average regressions for each method of incubation individually	80	.356193	.004452			
Remainder, difference between average regression coefficients for each method of incubation	1	.006839	.006839	1.54	3.96	6.96
With medium, all substrates studied versus without medium, excluding lima beans and okra:						
Deviations from pooled average regression for both methods of incubation	77	.294506				
Deviations from average regressions for each method of incubation individually	76	.293465	.003861			
Remainder, difference between average regression coefficients for each method of incubation	1	.001041	.001041	<1.00	3.97	6.99

[c] Reflects residual variation as measured from regression line or lines; error terms for variance ratio tests of significance are the mean squares shown for individual regressions.

[d] Variation among individual regression coefficients is significant; observed variance ratio, 3.36 exceeds its 1% tabular value, 3.17.

TABLE 4

F values for 10,000 spores of P. A. 3679 heated in some low-acid foods in TDT cans

Food	F value (minutes)	
	With medium	Without medium
Beef	13.9	10.0[e]
Pork	12.7	11.5[e]
Beans, lima	14.0	10.5[f]
Corn	12.9	10.0[f]
Peas	11.7	6.0[f]
Mushrooms	8.7	7.5

[e] Based on D value at 250°F (121.1°C.).
[f] From Reynolds, Kaplan, Spencer, and Lichtenstein (*6*).

TABLE 5

Calculated times for 90 percent destruction of 25,000,000 spores of Cl. botulinum 213-B heated in various substrates and incubated with medium supplementation

Substrate	Temperature (°F.)	D value	
		Stumbo's method	Schmidt's method
Beans, snap	240	.....	.28
	235	.53	.51
	230	1.01	.86
	225	1.62	1.96
	220	3.58	4.07
Beets	240	.48	.57
	235	.93	1.00
	230	1.09	1.17
	225	2.53	2.57
	220	5.01	5.65
Carrots	240	.30	.26
	235	.49	.40
	230	.....	.94
	225	2.46	2.46
	220	4.24	3.96
Corn	240	.68	.73
	235	.....	.75
	230	1.53	1.03
	225	.....	3.51
	220	8.30	8.46
Spinach	240	.....	.55
	235	.....	.83
	230	1.48	1.75
	225	2.91	2.91
	220	.....	6.68
Buffer[g]	239	.30	.38
	230	1.39	1.60
	225.5	1.62	1.79
	221	4.36	4.62
	212	20.73	23.49

[g] 200,000 spores, subcultured in tryptone yeast extract agar after heating.

TABLE 6

z and F values for 25,000,000 spores of Cl. botulinum 213-B heated in vegetable substrates and buffer and incubated with medium supplementation

Substrate	F value	z value			
		End point	Stumbo's method	Schmidt's method	Regression
Buffer[b]	.35	14.0	15.0	15.0	15.4
Beans, snap	.61	16.0	17.5	17.5	17.1
Beets	1.55	20.0	19.5	20.0	20.8
Carrots	1.25	20.5	17.0	17.0	15.9
Corn	2.25	20.0	18.0	16.0	17.9
Spinach	1.00	17.5	.....	18.5	18.4
Mean for vegetable substrates	.....	17.8	18.0	17.8	17.9[1]

[b] 200,00 spores.
[1] Obtained from covariance analysis.

Mean z values for thermal death time curves of *Cl. botulinum* in the vegetable substrates, when calculated by any of the proceeding methods, agreed well with the generally accepted z value of 18 reported for this organism *(2, 5)* while covariance analysis of the calculated z values showed no evidence of significant differences within the group at the 5% level and indicated that the mean calculated z value was representative of all the values. In all instances, F values for *Cl. botulinum* were lower than those found for P. A. 3679 heated in similar substrates and incubated with or without added medium.

DISCUSSION

The data presented in this paper lend further support to the use of a z value of 18 in calculating process times that are designed to sterilize low-acid foods, with respect to organisms such as putrefactive anaerobe 3679 or *Cl. botulinum*. Individual variations from z = 18 observed in this study with various substrates are within the range of experimental error. The data, therefore, provide no evidence to indicate that true z values varied with the substrate in which the spores were heated. It should be noted that findings of this investigation apply only to low-acid foods that have not been altered chemically to a significant degree by the addition of acids, preservatives, flavoring agents, or other compounds that might tend to vary the nature of the thermal death time curve. Where sufficient evidence, based on replicate determinations, shows that the z value for a particular food substrate is significantly different from 18, Gross, Vinton, and Stumbo *(3)*, then process time calculations should be based on the observed z value.

The fact that the calculated slopes of the thermal death time curves with okra and lima beans as substrates were significantly different from the other curves obtained without added medium does not invalidate the preceding conclusion. Rather, it emphasizes the greater reliability of observed z values when determinations are based on incubation of heated spores in food media made more suitable for germination and growth by addition of buffered peptone nutrients, as compared with recoveries determined by incubation in unmodified food substrates. As has been previously reported *(5, 6)*, some product inhibition is characteristic of most natural foods, i.e., heat treated spores unable to germinate and grow in such substrates and hence assumed dead, will develop when placed in

more suitable media. This point is further illustrated here by the data of Table 4 which show regularly higher F values (thermal death times) at 250°F.(121.1°C.) for P. A. 3679 spores when recovery was in food substrates modified with added peptone medium as compared with incubation in the unmodified food substrates. These results also indicate some product inhibition even with ground beef and pork which might be expected to provide excellent recovery media for putrefactive anaerobic bacteria. Poor or erratic growth of surviving organisms provides survival data showing increased variability. This is illustrated by the data of Table 1 where it will be noted that the standard deviation of z values based on incubation of heated spores in food substrates with added medium is approximately one-half that observed with incubation in the unmodified food substrates.

For these reasons the divergent z values observed for P. A. 3679 thermal death time curves in asparagus and okra when recovery was in the unmodified vegetable substrates may be assumed to represent the low side of a range of z values, if sufficient replicate determinations had been made. There is little reason to suppose that the mean of a sufficient number of replications would deviate significantly from the means of other substrates. A range in individual z values with any one specific substrate has been noted in the literature *(14)* and has also been observed in this laboratory, e.g. mushrooms unsupplemented with medium yielded a calculated z of 22 for this study as contrasted with a previously reported z of 16.5 *(6)*.

Further evidence indicating that observed variations in z values may be attributable to experimental error, is provided by a comparison of the data reported here with z values for thermal death time curves of P. A. 3679 in a large number of food substrates as reported in the literature. Table 7 lists the ranges and mean z values in different substrates as reported by 6 investigating groups. It is significant that over a period of years and with varying conditions of production of spores, size of inoculum, nature of substrate, recovery media and methods used in arriving at z values, the ranges and mean values (reported or calculated from the literature) are in close agreement with those reported in this study. Overall mean z value of the studies listed in Table 7 is 19.0, a value that also compares well with the mean values reported here, 18.7 with medium supplementation and 17.8 without medium supplementation.

It would appear from the preceding discussion that the needed laboratory thermal death time work for process time determinations based on the destruction of P. A. 3679, for the individual foods studied could logically be limited to runs at one temperature to position the thermal death time curve, z = 18, and to determine the F value from the positioned curve.

Data obtained with *Cl. botulinum* 213-B heated in several vegetable substrates indicate that processes calculated for P. A. 3679 will be more than adequate to destroy *Cl. botulinum* in these foods, thus confirming previous reports *(5, 6)*. F values for *Cl. botulinum* were lower in all instances than those reported in these studies and earlier *(6)*, for P. A. 3679 heated in similar substrates and incubated with and without medium supplementation. Statistical study of thermal death time data for *Cl. botulinum* and the mean z value of 17.9, calculated from covariance analysis,

TABLE 7

Ranges and average z values obtained from published reports for TDT curves of P. A. 3679 heated in low-acid food substrates

Source	Substrates	Range	Mean[1]
Tischer and Esselen *(13)*	Peas, snap beans, and corn brines	10.0-23.0	19.5
Reed, Bohrer, and Cameron *(5)*	Asparagus, green beans, green bean purée, frozen yellow corn, frozen yellow corn purée, fresh peas, frozen peas, pea purée, shrimp, spinach, spinach purée, sweet potatoes—7.5%, 30%, and 50% syrup	15.4-26.6	20.4
Sognefest, Hays, Wheaton, and Benjamin *(10)*	Asparagus, green beans, beets, carrots, white corn, peas, sweet potatoes, and pumpkin purées	16.0-21.0[a]	17.6[a]
Stumbo, Murphy, and Cochran *(12)*	Puréed peas, strained squash, puréed whole kernel corn, uncooked pork, cooked pork, white sauce, unsterilized evaporated milk, presterilized evaporated milk, unsterilized 20% cream	16.6-19.6	18.1
Townsend, Esty, and Baselt *(14)*	Asparagus, peas, spinach, whole milk	16.9-21.1	19.0
Reynolds, Kaplan, Spencer, and Lichtenstein *(6)*	Asparagus, lima beans, snap beans, beets, carrots, corn, mushrooms, okra, peas, sweet potatoes, pumpkin, spinach, summer squash (without added medium)	14.5-27.5	18.1
	Asparagus, snap beans, beets, carrots mushrooms, okra, sweet potatoes, pumpkin, spinach, summer squash (with added medium)	13.5-23.0	20.1
Grand mean			19.0

[1] Obtained as given by authors or calculated from their data.
[a] For foods at natural pH.

indicates the reasonableness of an assumption of z = 18 for the thermal death time curve of this organism.

SUMMARY

Statistical study of thermal death time data for P. A. 3679 heated in 15 low-acid foods in thermal death time cans and incubated with medium supplementation and in 13 low-acid foods without medium supplementation indicates that observed variation in individual z values may be attributed to experimental error rather than to the effect of specific food substrates. These results, along with results reported by other investigators, appear to justify the use of a z of 18 in process time calculations for the low-acid foods studied when they are not modified by the addition of compounds in concentration sufficient to affect the thermal destruction of P. A. 3679. Thermal death time data and statistical study of the z values obtained with *Cl. botulinum* 213-B heated in 5 vegetable substrates also confirm the validity of using a z of 18 for this organism and indicate that processes based on the destruction of P. A. 3679 in these foods will be adequate to destroy *Cl. botulinum* 213-B.

Acknowledgment

The authors are indebted to Elsie F. Dochterman and Albert B. Parks, statisticians, Bureau of Human Nutrition and Home Economics, United States Department of Agriculture, for the statistical evaluation of the data.

LITERATURE CITED

1. Ball, C. O. Mathematical solution of problems on thermal processing of canned foods. *Univ. Calif. Pubs. Public Health*, **1**, 15 (1928).
2. Esty, J. R., and Meyer, K. F. The heat resistance of the spores of *B. botulinus* and allied anaerobes. *J. Infectious Diseases*, **31**, 650 (1922).
3. Gross, C. E., Vinton, C., and Stumbo, C. R. Bacteriological studies relating to thermal processing of canned meats. VI. Thermal death-time curve for spores of test putrefactive anaerobe in meat. *Food Research*, **11**, 411 (1946).
4. Halvorson, H. O., and Ziegler, N. R. *Quantitative Bacteriology*. 1933, Burgess Publishing Company, Minneapolis, Minn.
5. Reed, J. M., Bohrer, C. W., and Cameron, E. J. Spore destruction rate studies on organisms of significance in the processing of canned foods. *Food Research*, **16**, 383 (1951).
6. Reynolds, H., Kaplan, A. M., Spencer, F. B., and Lichtenstein, H. Thermal destruction of Cameron's Putrefactive Anaerobe 3679 in food substrates. *Food Research*, **17**, 153 (1952).
7. Schmidt, C. F. (Personal communication, 1950).
8. Schmidt, C. F. (Personal communication, 1952).
9. Snedecor, G. W. *Statistical Methods*. 4th ed., 1946, Iowa State College Press, Ames, Iowa.
10. Sognefest, P., Hays, G. L., Wheaton, E., and Benjamin, H. A. Effect of pH on thermal process requirements of canned foods. *Food Research*, **13**, 400 (1948).
11. Stumbo, C. R. A technique for studying resistance of bacterial spores to temperatures in the higher range. *Food Technol.*, **2**, 228 (1948).
12. Stumbo, C. R., Murphy, J. R., and Cochran, J. Nature of thermal death time curves for P. A. 3679 and *Clostridium botulinum*. *Food Technol.*, **4**, 321 (1950).
13. Tischer, R. G., and Esselen, W. B., Jr. Home canning. I. Survey of bacteriological and other factors responsible for spoilage of home canned foods. *Food Research*, **10**, 197 (1945).
14. Townsend, C. T., Esty, J. R., and Baselt, F. C. Heat resistance studies on spores of putrefactive anaerobes in relation to determination of safe processes for canned foods. *Food Research*, **3**, 323 (1938).

EFFECTS OF LIGHT ON THE FORMATION OF CAROTENOIDS IN TOMATO FRUITS

J. P. McCOLLUM
Department of Horticulture, University of Illinois College of Agriculture, Urbana, Illinois

(Manuscript received July 30, 1953)

A number of investigators have studied the effects of light on the carotenoids of the tomato. In 1913, Duggar *(2)* showed that light was not essential for the formation of red pigment in tomatoes during ripening. Lubimenko *(3)*, in a study reported in 1916, maintained that light was not necessary for lycopene formation after chlorophyll has been formed. Smith and Smith *(6)* in 1931 and Veselkine *et al. (7)* in 1934 showed that neither light nor chlorophyll was essential for the formation of lycopene in tomato fruits. In 1935 Smith *(5)* found that, when light was excluded during development and maturity, carotenoids were decreased in the ripe fruit. A higher content of total carotenoids in the fruits of Golden Queen and Albino as a result of exposure to sunlight was cited by Smith as evidence for the effect of light on carotene. These fruits, however, contain lycopene as well as carotene which might have been affected by the light. In 1951, Denisen *(1)* found that green tomatoes from which light had been excluded were lower in carotene than the uncovered fruit.

Well-exposed tomatoes usually ripen to a yellow-red instead of a deep red color. This is generally true of the Earliana and other varieties with sparse foliage when grown in the field. In the greenhouse where the fruits are less exposed to sunlight this variety produces fruits of excellent color. The object of the present investigation was to determine the effects of sunlight and artificial light under controlled temperatures on the development of both lycopene and carotene in red tomatoes.

MATERIALS AND METHODS

Studies on the effects of sunlight were limited to differences in the exposure of field tomatoes. Detached fruits from both field and greenhouse were placed in constant temperature chambers with reflecting walls and illuminated from above by a bank of soft white florescent lights. Unless otherwise stated the fruits were exposed to continuous illumination of 400 F. C. as measured by a Weston illumination meter, model 756. Fans in the chambers prevented the temperatures of the illuminated fruits from rising above those of the surrounding air.

Samples were selected with regard to shading and each consisted of 12-15 fruits except where otherwise indicated. In all but one experiment fruits were analyzed a given number of days after incipient coloring (turning stage). Sampling error was further reduced in some tests by comparing the upper and lower halves of the same fruit. When whole fruits were compared, opposite quarters were taken for analyses. Total solids were determined by loss of weight on a 20-g. sample of juice at 80°C. in a ventilated oven. The method for total carotenoids and carotene was described in 1953 by the author *(4)*.

EXPERIMENTAL RESULTS

Ripe fruits of the Illinois T19 variety exposed to direct sunlight with central axes parallel to the ground were harvested for analyses. Sections of the pericarp approximately equal in size were taken from the upper and lower sides of 37 fruits. The pericarp from the 2 regions was measured for thickness and analyzed for total caro-

tenoids, carotene, and total solids. The data (Table 1) show the effects of sunlight and accompanying high temperatures on tomato fruits. Carotene and total solids of the pericarp were slightly higher on the exposed side of the fruit, but total carotenoids and thickness of pericarp were strikingly lower.

A well-shaded sample and one exposed to direct sunlight were harvested when fully colored and analyzed for total carotenoids and carotene. The shaded fruits were found to be slightly higher in total carotenoids but much lower in carotene than the fruits which had been exposed to direct sunlight during development and ripening, Table 2.

TABLE 1

Effect of sunlight exposure on tomato fruit pericarp

Side of fruit	Thickness	Total carotenoids	Carotene	Solids
	m.m.	*mg./pct.*	*mg./pct.*	*pct.*
Exposed	1.79	3.44	.800	6.30
Unexposed	3.39	11.36	.720	5.30

TABLE 2

Carotenoid content of shaded and unshaded fruits
Variety—Early Baltimore (9-12)

Treatment	Total carotenoids	Carotene
	mg. per 100 g. fresh wt.	*mg. per 100 g. fresh wt.*
Exposed fruits	7.32	.624
Shaded fruits	8.40	.390

Shaded and unshaded samples of Ill. T19 were harvested weekly at the turning stage and stored at 20°C. for 12 or 13 days. They were then analyzed for carotenoids, and the results for carotene are shown in Figure 1. The unshaded fruits were consistently higher than the shaded ones. There was also a decrease toward the end of the season except for the last harvest. At this time the plants were badly defoliated and the samples were more exposed than those harvested earlier.

A large number of green shaded fruits of the Rutgers variety were harvested September 30 after some cool rainy weather. They were divided into 2 lots, one of which was placed in an illuminated chamber and the other in an unilluminated one. Fruit temperatures in both cases were maintained at 24°C. Samples showing incipient color were selected on October 4 and analyzed 5 days later. The data (Table 3) show the illuminated fruits to be not only higher than the checks in carotene but almost 3 times as high in lycopene as indicated by total carotenoids.

The experiment was repeated with fruits harvested October 9 and analyzed October 16, 5 days after turning. The illuminated fruits were placed so that their central axes were perpendicular to the source of light. The upper and lower halves of these fruits were then analyzed separately. Total carotenoids as well as carotene were higher on

TABLE 3

Effect of light on the carotenoid content of green fruits harvested 9-30 and analyzed 10-9, 5 days after turning

Treatment	Temperature	Total carotenoids	Carotene
	Degrees C.	*mg. per 100 g.*	*mg. per 100 g.*
Exposed	24	11.80	.54
Unexposed	24	3.88	.39

Variety—Rutgers.

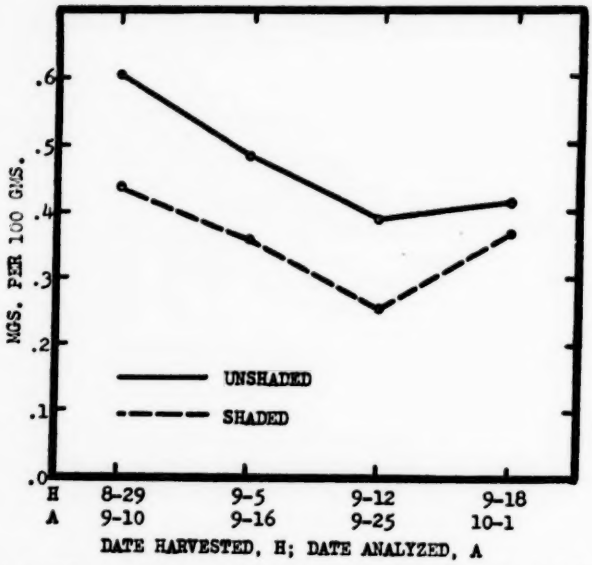


Figure 1. Effect of direct sunlight on the carotene content of tomato fruits.

the upper side of the fruit, Table 4. The fruits in the dark chamber were very low in total carotenoids and similar in carotene to the lower side of the illuminated fruits.

To determine the effect of light after the turning stage shaded samples of the Rutgers variety were harvested at the time of incipient coloring. One lot of fruit was placed in a lighted and another in an unlighted chamber. After 5 days both samples were analyzed. The experiment was repeated twice. The averages for 3 tests are given in Table 5. Although the data for total carotenoids are similar, those for carotene show a difference in favor of the illuminated sample.

The effectiveness of light after the turning stage was investigated further with mature green, shaded fruits. These were placed in an illuminated chamber with central axes perpendicular to the source of light. Fruits at incipient coloring were dated and divided into 2 groups. One remained in the light; the other was placed in the dark. After 5 days the upper and lower halves of the fruit in each sample were analyzed separately. There was an increase in total carotenoids in the upper as compared with the lower half of the fruit, Table 6. This difference was about the same in fruits illuminated until analyzed as in those illuminated only until the green color began to disappear. Carotene, on the other hand, was increased by light after the turning stage as in the previous experiment.

TABLE 4

Effect of light on the carotenoid content of green tomatoes harvested 10-9 and analyzed 10-16 five days after turning

Treatment	Half of fruit analyzed	Total carotenoids	Carotene
		mg. per 100 g.	*mg. per 100 g.*
Exposed	Upper	11.80	.60
Exposed	Lower	8.08	.32
Unexposed	Whole	2.24	.32

Variety—Rutgers.

TABLE 5

Effect of light exposure during ripening on carotenoid content
Variety—Rutgers

Treatment	Degrees	Wt. loss	Total carotenoids	Carotene
	C.	*pct.*	*mg. per 100 g.*	*mg. per 100 g.*
Exposed	25	6.27	5.56	.53
Unexposed	22	5.68	5.23	.32

Turning fruits analyzed after 5 days.
Average of three 12-fruit samples.

TABLE 6

Effect of light after the turning stage on the carotenoids of tomato fruits

Treatment after turning	Half analyzed	Total carotenoids	Carotene
		mg. per 100 g.	*mg. per 100 g.*
Exposed	Upper	7.67	.595
Exposed	Lower	5.78	.459
	Dif.(%)	32.70	29.63
Unexposed	Upper	7.47	.572
Unexposed	Lower	5.74	.504
	Dif.(%)	30.14	13.49

The effect of light on the pattern of coloring was determined with mature green, shaded fruits of Rutgers and Garden State. One-half of each sample was placed with stem ends up and one-half with flower ends up in an illuminated chamber. Five days after turning, the upper and lower halves of the fruits were analyzed separately for total carotenoids. The usual pattern of coloring from the apical to the basal end of the fruit was reversed by exposing the stem to the light, Table 7. Total pigments were similar in the 2 halves of Rutgers, but the basal half was much higher than the apical in Garden State. When the apical end faced the light this half was higher in pigments, but the difference was greater in Rutgers.

TABLE 7

Effect of light exposure after harvest on total carotenoids of tomatoes

Side exposed	Half of fruit analyzed	Variety	
		Garden state	Rutgers
		mg. per 100 g.	*mg. per 100 g.*
Stem end	Basal	8.48	7.40
Stem end	Apical	5.00	7.08
Flower end	Basal	9.80	8.48
Flower end	Apical	13.60	15.00

Shaded green fruits were harvested and placed in constant temperature chambers at different distances from the source of light to determine the effect of intensity of illumination on development of carotenoids. Samples of fruit with central axes perpendicular to the source of light were placed so as to receive respective light intensities of 0, 100, 200 and 400 F. C. At the time of incipient coloring the fruits were marked and 5 days later the upper and lower halves were analyzed separately for total carotenoids, carotene and total solids. The data (Figure 2) show the upper halves of the illuminated fruits to be higher in total pigments than the lower ones. On the basis of total carotenoids there is a downward trend with an increase in light intensity, but when the

data are expressed as percentage increase of the upper over the lower half of the fruit (Figure 3), both total carotenoids and carotene vary with intensity of illumination.

Tomatoes grown in the greenhouse and harvested as mature as possible without showing any red color were divided into 3 uniform lots to determine the effects of light on fruits with restricted gas exchange. The stem ends of 2 lots were waxed; one was illuminated and the other placed in the dark along with the unwaxed sample. When the latter was fully colored, all were analyzed for total carotenoids and carotene. The waxed sample in the absence of light remained green and was very low in carotenoids (Table 8). Most of the chlorophyll disappeared from the illuminated fruits, which were about one-half as high in total pigments and higher in carotene than the fruits that were neither waxed nor illuminated.

Other mature green samples were used to determine the effects of light on the coloring of fruits in an atmosphere without oxygen. Two samples were placed in sealed California petri dishes with open containers of alkaline pyrogallol. One was placed in the light, the other in the dark with an unenclosed sample. When the fruits of the latter were fully colored, each lot was analyzed and the data (Table 9) were found to be similar to those in the previous experiment. Both lycopene and carotene developed in illuminated fruits without atmospheric oxygen.

TABLE 8

Effect of light on the carotenoid content of excised tomato fruits with stem scars waxed

No.	Treatment	Total carotenoids	Carotene
		mg. per 100 g.	*mg. per 100 g.*
5	Waxed, no light	.30	.10
5	Waxed, light	3.68	1.04
5	No wax, no light	6.10	.66

TABLE 9

Effect of light on the carotenoid content of tomato fruits without atmospheric oxygen

No.	Treatment	Total carotenoids	Carotene
		mg. per 100 g.	*mg. per 100 g.*
3	Dark, without oxygen	1.00	.26
3	Light, without oxygen	3.80	.50
3	Dark, in air	6.68	.04

DISCUSSION

The poor color often observed in tomatoes exposed to direct sunlight is due primarily to a decrease in development of lycopene. The formation of this red pigment is apparently curtailed by the high temperatures of the exposed fruits. Although these fruits are higher in carotene than the unexposed ones, the parts of a fruit with color impaired by sunlight are only slightly higher in carotene than similar well-colored parts of the exposed fruit. Carotene may be increased by exposure of the developing fruit to sunlight as shown in Figure 1. The general downward trend toward the end of the season and the increase at the last harvest when the plants were partially defoliated were perhaps responses to the amount of illumination. The data indicate that field samples for comparison of color should be selected with regard to exposure during development as well as ripening.

When the temperatures were maintained at a range suitable for lycopene development, this pigment was increased by illuminating the fruit.

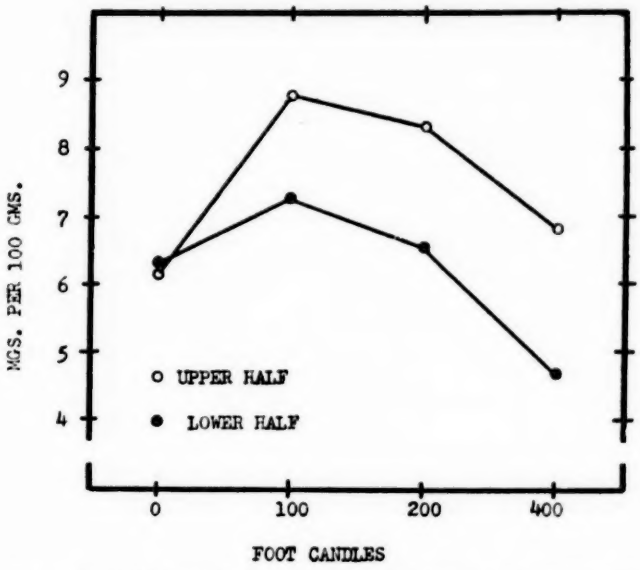


Figure 2. Carotenoid content of upper and lower halves of tomatoes exposed to different light intensities during ripening.

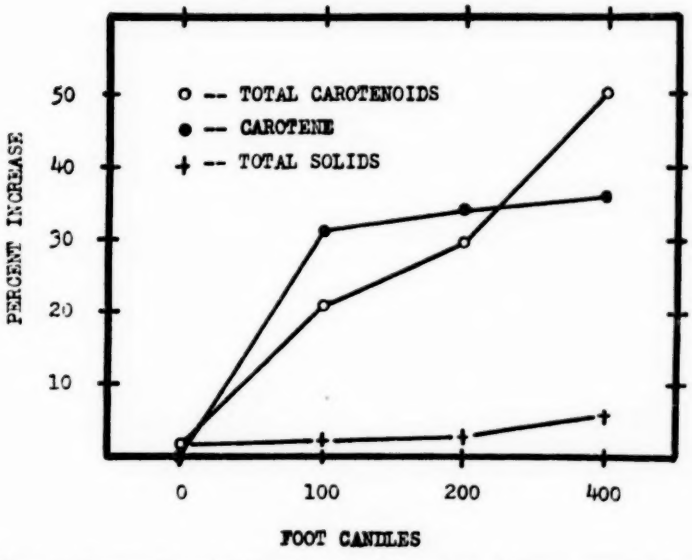


Figure 3. Increased carotenoid and total solids content of upper compared to lower halves of tomatoes exposed to varied amounts of illumination during ripening.

The results obtained (Tables 3 and 4) were very striking even in view of the fact that the fruits used in the experiments had been developing during cool and cloudy weather. The total pigment developed during the short exposure to light is about maximum for the variety.

There seems to be little effect of light on lycopene after a fruit begins to lose its green color. Carotene on the other hand apparently increases with light during ripening. The part of the fruit nearest the source of light begins coloring first. Thus, by exposing the basal end of the fruit to light the usual order of coloring from the apical to the basal end may be reversed. The more rapid coloring with proximity to light indicates that light intensity is a factor. When fruits were placed at different distances from the source of light and analyzed 5 days after the first appearance of color, there was a decrease in total pigments with increasing light intensity. This was due to the earlier initial coloring on the side of the fruit facing the light at the higher intensities. The effect of intensity is better shown by the difference between the upper and lower halves of the fruits, Figure 3.

The formation of lycopene has been found to depend upon the availability of atmospheric oxygen, Vogele (8). This was shown not to be true when tomato fruits were illuminated and maintained at a temperature range favorable for the production of lycopene. The skin of the tomato is impervious to the exchange of gases, causing a deficiency of oxygen in the fruit during ripening. The increase in rate of pigmentation of fruits in a normal atmosphere by the use of lights may thus result to some extent from oxygen supplied through the action of photosynthesis.

SUMMARY

Tomato fruits exposed to direct sunlight in the field often develop poor color due primarily to a low content of lycopene. At favorable temperatures (70-75°C.) the rate of development of this pigment is increased by illumination. Light is effective until chlorophyll disappears.

Carotene is increased by illumination of tomato fruits during ripening. Also, if exposed to direct sunlight during development, the fruits will be higher in carotene when ripe than shaded fruits.

The chlorophyll of mature green tomatoes does not decompose and the carotenoids fail to develop in the absence of atmospheric oxygen unless the fruits are illuminated. Oxygen is apparently supplied by photosynthetic action, which in view of the impervious skin of the tomato may partially explain the effects of light on carotenoid development in a normal atmosphere.

Exposure of the fruits to light during both development and ripening is an important factor in the selection of comparable samples for comparison of color.

LITERATURE CITED

1. Denisen, E. L. Carotenoid content of tomato fruits as influenced by environment and variety. I. Effect of temperature and light. *Iowa State Col. Jour. of Sci.*, 25, 549-564 (1951).

2. Duggar, B. M. Lycopersicum, the red pigment of the tomato, and the effects of conditions upon its development. *Wash. Uni. Studies*, 1 (1), 22-45 (1913).

3. LUBIMENKO, W. Transformations of plastid pigments in living plant tissue. *Acad. Sci. Petrograd. Mem.*, Ser. 33, 1-275 (1916). (*Chem. Abstr.* **14**, 1697-1698, 1920.)
4. MCCOLLUM, J. P. A rapid method for determining total carotenoids and carotene in tomatoes. *Am. Soc. Hort. Sci. Proc.*, 1953 (in press).
5. SMITH, ORA. Effects of light on carotenoid formation in tomato fruits. N. Y. (Cornell) Ag. Exp. Sta. *Mem.* 187 (1936).
6. SMITH, LAURA LEE W., AND SMITH, ORA. Light and the carotenoid content of certain fruits and vegetables. *Plant Physiol.*, **6**, 265-275 (1931).
7. VESELKINE, N. V., LUBINIENKO, V. N., BULGAKOVA, Z. P., TIKAL'SKAIA, V. V., AND ENGEL, P. S. Influence de la lumiere sur la synthese des vitamines. *Izv. Nauchn. Inst.* P. F. Lesgafta. *(Bull. Inst. Sci. Lesshaft)* **17/18**, 389-404 (1934).
8. VOGELE, A. C. Effect of environmental factors upon the color of the tomato and the watermelon. *Plant Physiol.*, 12, 929-955 (1937).

RAW STARCH DIGESTION: A COMPARISON OF THE RAW STARCH DIGESTING CAPABILITIES OF THE AMYLASE SYSTEMS FROM FOUR ALPHA-AMYLASE SOURCES[a]

R. M. SANDSTEDT AND R. L. GATES
University of Nebraska College of Agriculture, Lincoln, Nebraska

Manuscript received August 26, 1953

Little is known concerning the enzymes responsible for the digestion of raw starch. It has been shown that β-amylase does not digest raw undamaged starch *(12, 15)*, which leaves α-amylase as the most probable agent. However, it also has been hypothesized that a special factor may be responsible for this action *(1)*.

Recent reviews comparing the properties of the amylases from various sources *(6, 7)* indicate a wide variability in the properties of the α-amylases between sources. Classification of the enzymes according to source (such as fungals, bacterials, etc.,) carries the implication that the enzymes not only vary with the class of organism from which they originate but also that those from related organisms are similar. However, though the enzymes from one class of organism have characteristics enough in common to justify such classification, the α-amylases from related sources do vary *(8)* and should not be categorically considered alike.

Most of the studies that have included a determination of enzymatic attack in raw starch have approached the problem from the point of view of the relative susceptibility of different starches. The wide variation in susceptibility was well known to the early researchers as indicated by Reichert's *(11)* extensive review. Recently Booher, Behan and McMeans *(2)* studied the relative susceptibility of raw starches to digestion in the rat gut. Yamazaki and Ueda *(16)* determined the relative susceptibility of 14 different starches to attack by the "black koji" amylase. These studies confirm the earlier work showing that the raw cereal starches were the most easily digested and the raw root starches were the most difficult to digest.

There has been little evidence presented of the relative abilities of enzymes from various sources to digest raw starch. If the α-amylase is the component responsible for the digestion of raw starch it would seem probable that its action should correlate with the raw starch digesting action of a series of enzyme systems. However, a lack of correlation would not necessarily prove that the α-amylases are not the active agents but may merely indicate another variable in the action of the α-amylases.

MATERIALS AND METHODS

Enzyme sources. Four distinct classes of enzyme source—7 fungals, 3 bacterials, 3 pancreatics, and 3 malts—were used in the preliminary survey for this study. With the exception of an acetone precipitated barley malt preparation these were enzyme concentrates furnished by eight different commercial firms. For further study, one con-

[a] Published with the approval of the Director as Paper No. 622, Journal Series, Nebraska Agricultural Experiment Station.

centrate of each type of enzyme source was selected: B8-Takamine bacterial No. 3319, P3-Takamine pancreatic No. 4373, F7 Takamine "Clarase" No. 4183, and M5-Columbia Malting Company "Colase 1000."

Starch substrate. The raw starch substrate was a sample of commercial corn starch.

Determination of alpha-amylase. The determination of α amylase was by the method described by Sandstedt, Kneen and Blish *(13)*. The method was modified by using the glass color-standard as suggested by Redfern *(10)* and the "β amylase for analytical purposes" from the Wallerstein Laboratories.

Determination of raw starch digestion. The method used to determine raw starch digestion was that described by Gates and Sandstedt *(4)*. Essentially the method consists of a 20-hour digestion at 30°C. with constant stirring of 0.5 g. of raw starch in 20 ml. of a solution containing the enzyme and buffer. The undigested residue is filtered, dried and weighed. The loss in weight is the measure of enzyme activity.

RESULTS

Preliminary survey of alpha-amylase sources. The raw starch digesting potentialities of two or more preparations of each of the sources of α amylase were studied under the conditions found to be optimum for one of them, B6 (4); a 20-hour digestion at 30°C. buffered at pH 6.5 with M/15 phosphate with 0.1% "Rocceal" added to prevent microorganism growth.[b]

Gates and Sandstedt *(4)* define the unit of raw starch digestion as that enzyme activity required to accomplish 20% digestion of one mg. of raw corn starch in 20 hours at 30°C. Accordingly, digestions were carried out at a sufficient number of concentrations, two or more, so that it was possible by graphing and interpolation to determine the quantity of enzyme which would be required to accomplish 20% digestion of one gram of raw corn starch (1,000 units).

The survey indicated that in general the raw starch factor was to be found in high concentration in bacterial and pancreatic preparations whereas the malt and fungal preparations were only 5% to 10% as effective. Based on the α amylase content of the preparations, the malt and bacterial concentrates were 10 to 20 times as effective, and the pancreatic sources about 100 times as effective as the fungal preparations under these conditions of buffer, pH and Rocceal activation.

Relation between enzyme concentration and extent of raw starch digestion. Since these comparisons were based on a pH which was optimum for B6 but not necessarily for the enzymes from other sources, one of each of the four types of α amylase source was selected for further study. The effect of variations in pH adjusted with M/15 phosphate for each of the enzyme concentrations was determined. Under these conditions with a digestion period of 20 hours at 30°C., it was found that the hydrogen ion concentrations for maximum digestion were pH 6.6 for the first two and pH 4.5 and 4.1, respectively, for the last two. However, because of a lack of an established relation between the enzyme concentration and the extent of starch digestion it was difficult to make exact comparisons of activity over the entire pH range studied. The starch-enzyme ratio was not constant throughout these pH studies.

To establish this relation between enzyme concentration and the extent of raw starch digestion, an extract of each of the preparations was made which was of such a concentration that 10 ml. would give approximately 50% digestion. Two-, 5-, 10-, and 15-ml. aliquots of these extracts were permitted to digest raw corn starch in M/15 phosphate buffer at the pH of maximum activity for the individual enzyme and in the presence of 0.1% Rocceal. The results of this study presented in Figure 1 show the wide variation in raw starch digesting ability of the preparations. The pancreatic and bacterial preparations carried high concentrations of the raw starch factor. This figure also illustrates how the raw starch digesting values in units *(4)* were calculated from such curves. (However, when used for determining enzyme activity values, the enzyme concentration should be graphed on an extended scale in order to give accurate readings for the more active preparations.)

A different picture of the action of the raw starch factors is obtained if their action is compared on a raw starch unit basis (Figure 2). These curves show that each of

[b] "Rocceal" is not recommended for this purpose; it has been found to have varied activating and inhibiting actions under differing conditions.

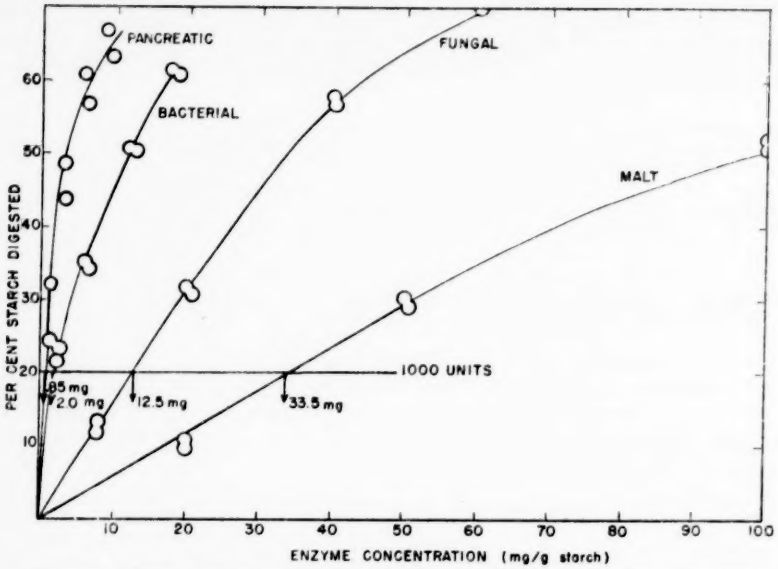


Figure 1. Comparison of raw starch digestion by enzyme concentrates. Illustrating the method of determining the quantitative raw starch digesting unit.

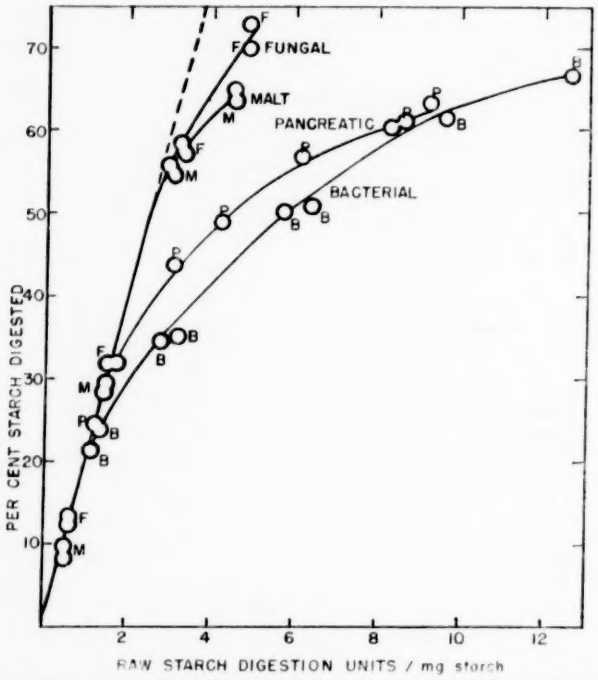


Figure 2. Raw starch digestions compared on a raw starch digestion unit basis. The broken line indicates the hypothetical linear relation.

the enzyme systems had its own distinct pattern of action on raw starch. The relation between enzyme concentration and extent of starch digestion was practically linear up to 50% digestion for the M5 and F7 enzymes, whereas B8 and P3 showed a deviation from a linear relation above 20% digestion. (However, by definition of the raw starch unit all were made equal at the 20% digestion level; accordingly the deviation noted probably exists from the beginning of the digestion.) Apparently, under the conditions used, the raw starch factors of the bacterial and pancreatic preparations were more subject to inhibition than those of the malt and fungal preparations.

Influence of pH on digestion. With the establishment of a relation between extent of starch digestion and quantity of enzyme, it is possible to present the results of the study concerning the effect of pH on digestion. The pH values were obtained with M/15 phosphate. The digestion period was 20 hours at 30°C. and with and without 0.1% Roccal present.

The results are presented in Figures 3, 4, 5 and 6. As was reported earlier, the maximum activity with B8 and P3 was obtained at or near a pH of 6.6. With the M5 and F7 concentrations the pH values for maximum digestion were near 4.5 and 4.1, respectively. In the presence of Roccal the action on raw starch was more sensitive to variations in pH than is α amylase action. In the absence of Roccal there was markedly less sensitivity to pH change, except in the case of malt.

Stimulation of raw starch digestion by Roccal. Because of the stimulating effect of Roccal on the digestion of raw starch by a pancreatic amylase (*3*), it was deemed desirable to ascertain the effect of this agent on the digestion of raw starch by the

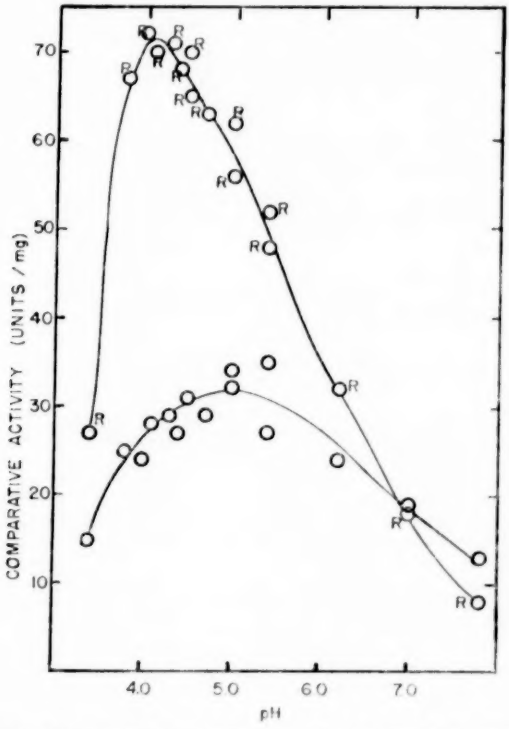


Figure 3. The pH-raw starch activity curves for a fungal enzyme system (F7). M/15 phosphate buffer with and without 0.1% Roccal (R).

four selected enzyme concentrates. The stimulating and inhibiting effects are presented graphically in Figures 3, 4, 5 and 6.

There was a considerable difference between the enzyme systems in their response to Rocecal. However the increased digestion at the pH for maximum action for the F7, B8 and P3 enzymes was similar, about 2.3 times. This quantitative similarity for 3 such differing enzyme systems suggests that the Rocecal action was on the starch, not on the enzymes. The pH for maximum activity for F7 differed in the presence and absence of Rocecal. Without Rocecal the optimum pH was about 5.0, whereas with this agent the optimum pH was changed to 4.1.

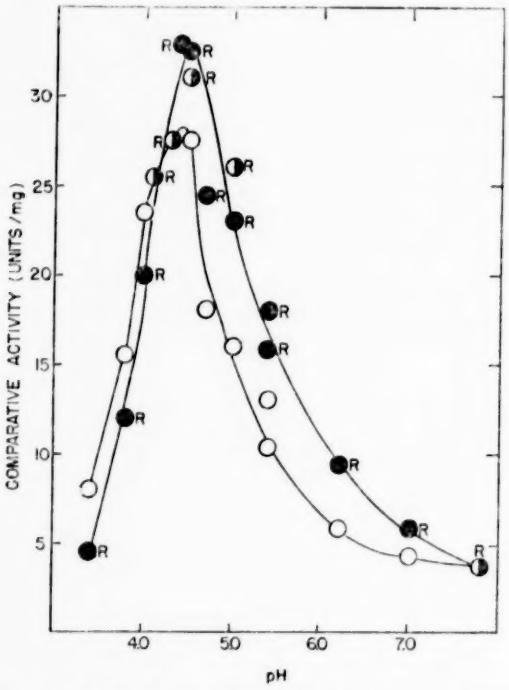


Figure 4. The pH-raw starch activity curves for a malt enzyme system (M5). Conditions as in Figure 3.

With malt as a source of enzyme (Figure 4), Rocecal caused inhibition of action at the lower pH values and gave little stimulation at the optimum or at pH's above the optimum. The optimum pH for activity was independent of the presence of Rocecal.

The B8 (Figure 5) and P3 (Figure 6) enzymes were quite similar in their response to Rocecal, both indicating inhibition at lower pH's with a maximum stimulation at the optimum pH for activity. The optimum pH was independent of the presence of Rocecal.[c]

A comparison of alpha-amylase and raw starch digestion activities. The preliminary survey comparing the raw starch digesting potentialities of the enzyme sources with their α-amylase values indicated that the ratio of these two values varied widely depending on the type of organism from which the enzymes originated. However, the pH's

[c] Further investigation of the Rocecal effect indicates that the increased digestion occurs only in the presence of phosphate (buffers). With other buffers Rocecal is an inhibitor of raw starch digestion.

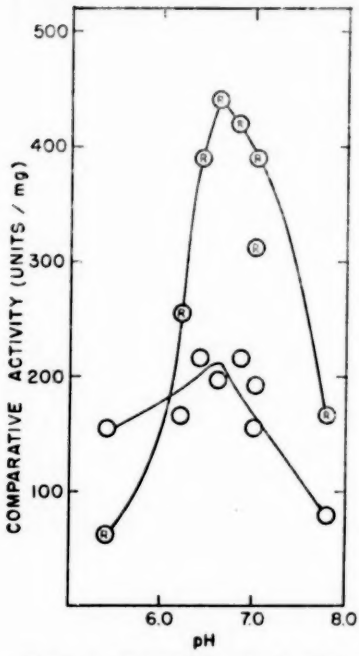


Figure 5. The pH-raw starch activity curves for a bacterial enzyme system (B8). Conditions as in Figure 3.

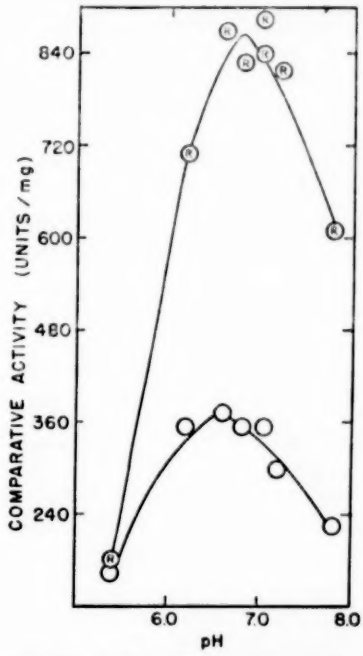


Figure 6. The pH-raw starch activity curves for a pancreatic enzyme system (P3). Conditions as in Figure 3.

used were not optimum for the individual enzyme systems. Accordingly, these comparisons were repeated on the four selected enzyme systems using in each case optimum pH values for the α-amylase and raw starch digestions. The data, presented in Figure 7, show that under optimum conditions for digestion, based on equal α-amylase activity, B8 was 4 times, M5 was 6 times, and P3 was 20 times as effective as the F7 preparation for the digestion of raw corn starch.

The relation between the amount of starch solubilized and the fraction which can be accounted for as reducing sugar. In the preceding studies the amount of activity was determined not only by disappearance of insoluble starch but by an analysis of the filtrate for the total reducing value, calculated as maltose (maltose-equivalent). For each digestion the ratio of maltose-equivalent to starch loss (the reducing value of the end products of the digestion) was calculated. These ratios, which represented more than 50 determinations for each enzyme, approached a constant value characteristic of the individual enzyme preparation. The standard deviation for these values was 0.05. The characteristic values for each preparation were B8—0.71, P3—0.75, M5—0.95, and F7—1.15.

The marked uniformity of these values for an individual enzyme source—over a wide range of conditions of digestion including variations in pH, enzyme concentration, and the presence of activating and inhibiting salts—indicates a comparatively stable and characteristic pattern of action for each enzyme system. The values below 1.00 may be interpreted as incomplete conversions of dextrins to maltose. The values for the malt enzyme system are within experimental error of 100% conversion. The high value for the fungal preparation indicates the production of both maltose and glucose *(14)*—this has been confirmed by paper chromatography.

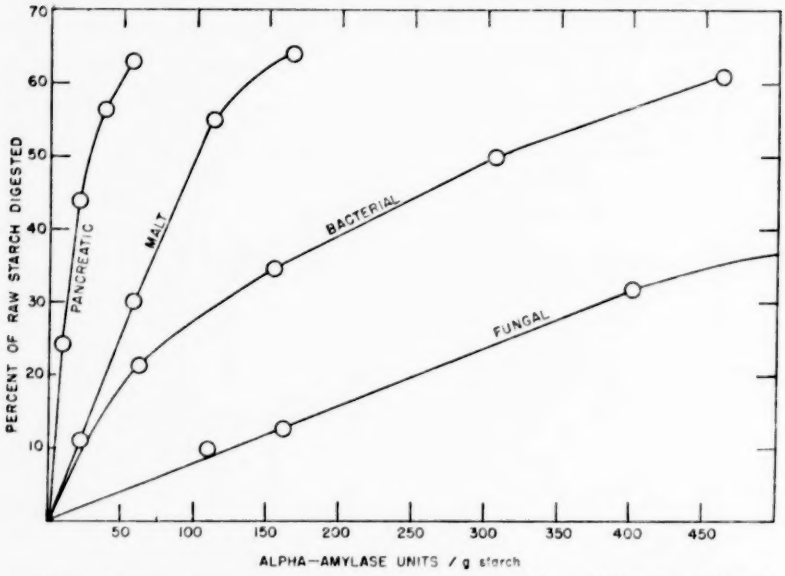


Figure 7. Comparison of α-amylase and raw starch digestions. M/15 phosphate buffer at the pH for maximum activity for each enzyme system. Roccal (0.1%) present in the raw starch digestions.

However, there were some conditions of digestion under which these reducing value ratios were altered. The most significant of these were with M5 and F7 in the absence of Roccal at the higher pH values. The ratios for these digestions were normal at pH 5.8 but dropped to 0.53 and 0.41, respectively, at a pH of 7.8. It is interesting that this response should be observed in the absence of Roccal but not when it was present during the digestion period. Roccal also seems to activate the factor in the enzyme system of F7 which is responsible for raising the ratio above 1.00. In digestions with this enzyme run side by side, those which took place in the presence of Roccal gave ratios which were about 0.20 higher than those which took place in its absence.

DISCUSSION

A comparison of the raw starch digesting systems of the four sources of α-amylase gives evidence of five differentiating properties of these systems: The optimum pH for attack of raw starch, the stimulation of raw starch digestion by Roccal, the maltose-equivalent of the products of starch digestion, the ratio of raw starch digestion to α-amylase and the per cent of starch digested by high concentrations of raw starch factor (more than 2 units per mg. of starch).

The concept of α-amylases of different sources having distinct and characteristic pH optima for activity is not new. In general the pH optima exhibited by these different sources for raw starch digestion are similar to, but more critical than, those reported for α-amylase activity of the same sources. The F7 enzyme was peculiar in that its optimum pH was changed by the addition of Roccal.

The marked stimulation of raw starch digestion accompanying the addition (under specific conditions) of the cationic detergent, Roccal, is an interesting point of difference between raw starch digestion and α-amylase action. This particular phase of the starch digestion problem is under investigation. A relationship has been suggested between the increased rate of action of pancreatic amylase in the presence of Roccal and the greater efficiency reported for feeds containing certain detergents *(3)*. The response of the enzymes from different sources to this organic electrolyte served as a means of differentiation. In the case of the malt enzymes, little response was observed, whereas the enzymes from other sources have their activity on raw starch materially augmented. This indicates that the malt enzymes attack the starch granule in a different manner.

The difficulty of measuring enzymic activity on raw starch by the quantity of reducing materials produced is evident from the results reported here. The fraction of the total starch solubilized which could be accounted for as maltose was markedly uniform for any one source of enzyme. The relatively incomplete conversion observed with the P3 and B8 enzymes and complete conversion experienced with malt and the F7 enzymes agrees with the characterization of similar enzymes reported by Pigman *(9)*. He found that when the enzymes digested a cooked starch substrate in the presence of yeast, the malt and fungal enzymes gave complete conversion of the starch to fermentable materials whereas the other two sources gave incomplete conversion. How well the various individuals of each group compare is still to be seen—the question of whether most of the fungals fit into a fungal pattern, bacterials into a bacterial pattern, etc., has not been shown.

One of the main objectives of the research was to determine whether there was a relation between the α-amylase activity of any given material and the capacity of that material to digest raw starch. It is possible to assign a measure of α-amylase activity to an enzyme source, but can that designation be used as a clue to the ability of that source to digest raw starch? Among representatives of any one type of α-amylase it may be possible to use the α-amylase activity as an indication of the relative capacity of these preparations to digest raw starch. However, when two or more types of source are compared, there is no correlation.

When a direct comparison was made of the activity of individual enzyme sources on raw and on cooked starch it was found that, on the basis of α-amylase content, the enzymes may be from 165 to 7,000 times as active on the cooked starch as on the raw starch. This wide variation in activity may be related to the affinity of the enzymes for the starch substrate as discussed by Hopkins *(5)* concerning the action of the enzymes on cooked starch substrates.

From this study of raw starch digestion it is evident that there are few properties which are common to all α-amylase sources. This series of studies was originally designed to determine if there was any similarity in the pattern of action of the enzymes of various α-amylase sources in their attack on raw starch. Had it been possible to show from these studies that by knowing the α-amylase content of a preparation it would be possible to predict the extent of action of the preparation on raw starch, it would be

good evidence that *a*-amylase and the raw starch digesting enzyme are the same.

However, these studies show that the enzymes from the different sources vary greatly in their relative *a*-amylase activities and capacities to digest raw starch. This does not preclude the possibility that in preparations of any one type of *a*-amylase, similarities would be found. The differences observed may be attributable to fundamental differences in the properties of the enzymes responsible for the *a*-amylase type activity, or the enzyme responsible for the digestion of raw starch may not be *a*-amylase; this will be determined by the use of purified *a*-amylases from the various sources.

SUMMARY

There is a wide variation in the relative potential of amylase systems from various sources to digest raw starch. A comparison of malt, fungal, bacterial and pancreatic enzyme systems at a constant level of *a*-amylase activity showed that their ability to digest raw starch was not proportional to the *a*-amylase. Used on the *a*-amylase basis, the pancreatic enzyme system was the most effective in digesting raw starch, followed in order by the malt, the bacterial and the fungal; the pancreatic was 20 times as effective as the fungal. The optimum pH of each of the enzymes for raw starch digestion corresponded to that observed for the *a*-amylase activity of the particular system. The reducing value of the end products of raw starch digestion was different for each of the enzyme preparations approaching a constant value for any one preparation. The action of these preparations on raw starch was affected differently by the presence of a cationic detergent, "Rocceal."

LITERATURE CITED

1. Blish, M. J., Sandstedt, R. M., and Mecham, D. K. Action of wheat amylases on raw wheat starch. *Cereal Chem.*, **14**, 605-628 (1937).
2. Booher, Lela E., Behan, Ida, and McMeans, Evelyn. Biologic utilization of unmodified and modified food starches. *J. Nutrition*, **45**, 75-100 (1951).
3. Gates, R. L. and Sandstedt, R. M. Effect of a cationic detergent on the digestion of raw corn starch in vitro. *Science*, **116**, 482-483 (1952).
4. Gates, R. L., and Sandstedt, R. M. A method for determining enzymatic digestion of raw starch. *Cereal Chem.*, **30**, 413-419 (1953).
5. Hopkins, R. H. The action of the amylases. *Advances in Enzymology*, **6**, 388-414. 1946, Interscience, New York.
6. Kneen, E. The amylases. Properties and production. Chemistry and Industry of Starch. R. W. Kerr, ed., 1950, Academic Press, Inc., 407-441.
7. Myrback, K., and Neumuller, G. Amylases and the hydrolysis of starch and glycogen. *The Enzymes*. Ed. by J. B. Sumner and Karl Myrback, 1950, Academic Press, Inc., 653-724.
8. Okazaki, H. Amylase systems of various molds. *J. Agr. Chem. Soc., Japan*, **24**, 88-96 (1950).
9. Pigman, W. W. Extent of hydrolysis of starches by amylases in the presence and absence of yeasts. *J. Res., Natl. Bureau Standards*, **33**, 105-120 (1944).
10. Redfern, Sutton. Methods for the determination of alpha-amylase. IV. A glass end point color standard for use in the dextrinizing method; effect of temperature and starch lot on this method. *Cereal Chem.*, **24**, 259-268 (1947).
11. Reichert, E. T. The differentiation and specificity of starches in relation to genera, species, etc. Part I. Carnegie Institute of Washington, 173 (1913).

12. Sandstedt, R. M., Blish, M. J., Mecham, D. K., and Bode, C. E. Identification and measurement of factors governing diastasis in wheat flour. *Cereal Chem.*, **14**, 17-34 (1937).
13. Sandstedt, R. M., Kneen, Eric, and Blish, M. J. A standardized Wohlgemuth procedure for alpha-amylase activity. *Cereal Chem.*, **16**, 712-723 (1939).
14. Schwimmer, Sigmund. The role of maltase in the enzymolysis of raw starch. *J. Biol. Chem.*, **161**, 219-234 (1945).
15. Stamberg, Olaf E., and Bailey, C. H. Studies on wheat starch. II. The action of amylase on raw wheat starches. *Cereal Chem.*, **16**, 319-330 (1939).
16. Yamazaki, Izue, and Ueda, Seinosuke. Action of black-koji amylase on raw starch. *J. Agr. Chem. Soc., Japan*, **24**, 181-185, (1951). (*Chem. Abst.*, **45**, 9089.)

DENATURATION OF MYOSIN DURING DEFROSTING OF FROZEN FISH

OLAVI E. NIKKILÄ AND REINO R. LINKO
Central Laboratory of Tukkukauppojen Oy, Turku, Finland

(Manuscript received August 28, 1953)

Changes in the quality of freshly caught fish are chiefly caused by autolysis and bacterial decomposition. The rate of autolysis and spoilage depends very much on the temperature. Freezing prevents or at least delays these processes and thus frozen fish can be kept in good condition for long periods at low temperatures. However, freezing and subsequent storage may effect changes of another kind which are undesirable and may make the fish less palatable. These changes vary depending on the species, the freshness and pretreatment of the fish, the freezing methods, and storage conditions. One of the deteriorations in question involves primarily an irreversible change in the protein fraction of frozen fish. This change results in a drier muscle with a coarser texture. The cause of the change seems to be a denaturation of the proteins, which lose their ability to swell and retain the muscle juice and to return to their original condition after defrosting. No complete explanation of fish protein denaturation caused by freezing is forthcoming at present in spite of the numerous investigations conducted in these fields, Reay *(9, 10)*, Reay and Kuchel *(11)*, Notevarp and Heen *(6, 7)*, Dyer, French and Snow *(3)*, Snow *(12)*, and Dyer *(2)*.

The conversion of native protein to denaturated protein can be brought about by various means, for example, by a high or low temperature. A protein denatured so that it is insoluble in water or in dilute salt solution undergoes a change in a number of its other properties. In this investigation the term denaturation has been used to denote loss in brine extractibility of the myosin fraction. By electrophoresis, by ultracentrifugation and by other methods this fraction has been subdivided further. Our present view of the fundamental nature and behavior of these substances is primarily based on the work of the Szent-Györgyi and Dubuisson schools. Actually, however, the results and conclusions of these workers are not uniform in all details. Furthermore, the nomenclature of muscle proteins is confusing. For convenience, the fraction extracted by a salt solution and precipitated on dilution with water is called myosin in this paper.

METHODS

The soluble proteins from fillets of Baltic herring *(Clupea harengus* var. *membranus)* were extracted at 0°C. with a 5% sodium chloride solution according to the method described by Dyer, French and Snow *(3)*. The myosin present in the brine extract was precipitated by diluting 10-fold with distilled water at 0-5°C. After standing overnight in the refrigerator, the myosin was centrifuged off and the myosin nitrogen was determined by the micro-Kjeldahl modification described in the A.O.A.C. *(1)*. In agreement with the results of Hamoir *(4)*, no myosin was observed in the electrophoretic pattern of extracts dialyzed against a buffer of ionic strength, $\mu = 0.11$ and pH 7.2, and therefore the precipitation of myosin was considered complete. Total nitrogen and soluble

nitrogen were estimated by the Kjeldahl-Gunning-Arnold method. Nonprotein nitrogen was determined by the same method after precipitation of the protein from a 5% trichloracetic acid solution. Nonmyosin nitrogen was obtained by subtracting myosin nitrogen from soluble protein nitrogen.

Fish packed in waxed cartons or in pliofilm bags were frozen in an air blast at —35°C. and stored subsequently at —20°C. The frozen fish were allowed to defrost under various conditions. The details of defrosting and subsequent storage are given together with the experimental results in Table 1 and Figures 1-3.

RESULTS

Table 1 shows the effect of the temperature and time of defrosting on the extractibility of proteins. A low defrosting temperature and short time lead to no considerable decrease in the extracted proteins, but high temperature and longer storage after defrosting diminish to a great extent the amount of proteins extracted by the salt solution.

The curves in Figure 1 illustrate the changes occurring in the amounts of protein extracted by brine from fish muscle during freezing, cold storage and defrosting. The curves designated I refer to fish that were frozen in fresh condition immediately after the resolution of *rigor mortis* and the curves designated II to fish frozen in *rigor mortis* condition. In the first case the amount of myosin nitrogen is as great in muscle frozen at —35°C. and kept at —20°C. for 12 days as in fresh muscle before freezing. During defrosting at 18°C. in air, the myosin nitrogen curve I runs almost linear for about

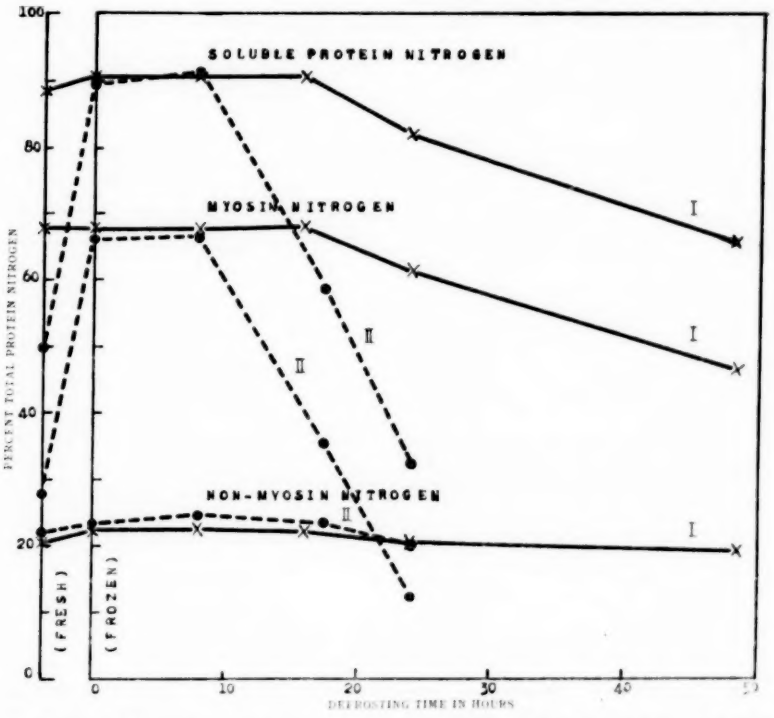


Figure 1. Changes in the amount of myosin nitrogen present in brine extracts from fish muscle during freezing, cold storage and defrosting. Curves I refer to fish frozen immediately after resolution of rigor mortis, curves II to fish frozen during rigor mortis.

TABLE 1

The variation of extractable protein nitrogen when frozen fish are defrosted under various conditions

Defrosting and storage					
In water			In air		
Temperature	Time	Soluble protein N/total protein N	Temperature	Time	Soluble protein N/total protein N
°C.	hrs.	%	°C.	hrs.	%
Before freezing		91.9	....	....	91.9
5	3	90.2	18	8	90.2
20	1	86.5	18	21	81.8
20	3	84.1	18	27	55.8
30	½	79.0	18	41	48.6
50	¼	33.9	....	....	....

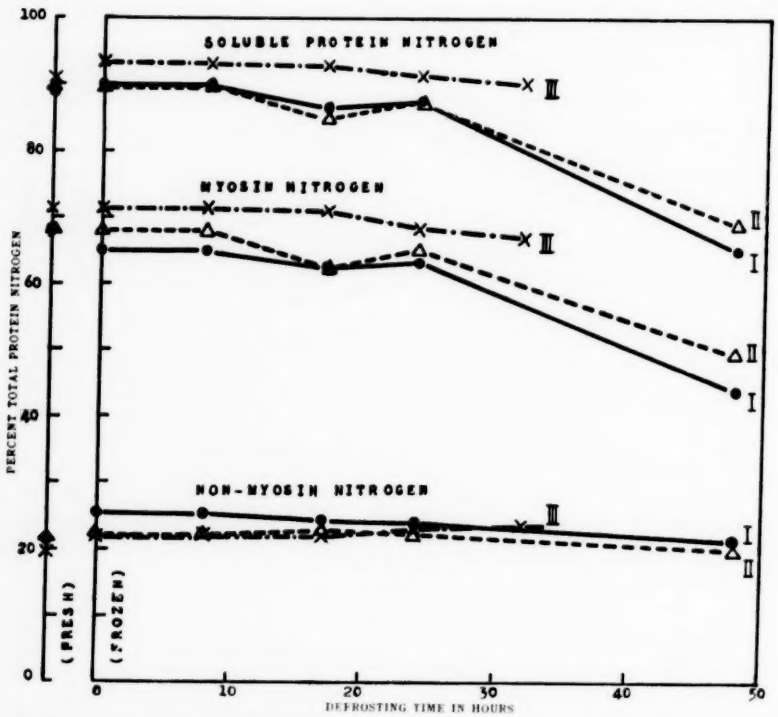


Figure 2. The variation during defrosting of the amount of myosin nitrogen extracted by brine from frozen fish muscle. Curves I refer to fish frozen in the fresh condition, curves II to fish stored for 34 hours and curves III to fish stored 58 hours at 18°C. before freezing.

17 hours. After this time it gradually lowers. The myosin nitrogen extracted from *rigor mortis* muscle was only 28% of total protein nitrogen as compared with 68% in fresh muscle after the resolution of *rigor mortis*. During storage in frozen condition *rigor mortis* resolves slowly and the extractable myosin increases. Chilling and freezing prolong the final resolution of *rigor mortis*, but are not able to inhibit it. Evidently owing to some type of interaction of proteins in *rigor mortis*, the myosin in fish frozen in this condition more readily undergoes a change during subsequent cold storage which results in a lowered extractibility on defrosting than in the case of fish frozen after the resolution of *rigor mortis*. This opinion is supported by the observation that the myosin is denatured during a much shorter defrosting period in the first case than in the second.

The curves in Figure 2 show the effect of storage at 18°C. for 10 hours (I), 34 hours (II) and 58 hours (III) before freezing on the amount of the protein nitrogen in the brine soluble fraction. Storage at 18°C. before freezing increases the extractibility of myosin; this is evidently due to autolysis. When the fish are subsequently defrosted in air at 18°C., no loss in the soluble myosin fraction occurs during 7 hours, but after this period the brine solubility of myosin begins to decrease. During defrosting and storage for two days in air at 18°C., the amount of myosin nitrogen in fresh frozen fish (curve I) decreases from 65% of the total protein nitrogen to 43%. The myosin fraction extracted by brine from fish stored for some time before freezing (curves II

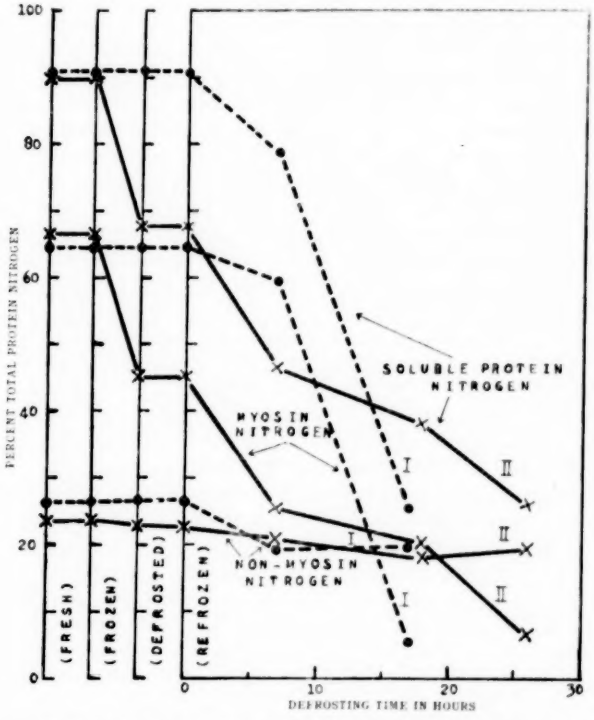


Figure 3. Changes in the amount of myosin nitrogen extracted by brine from fish muscle during freezing, defrosting, refreezing, and defrosting. Curves I refer to fish which were defrosted the first time 8.5 hours at 18°C. Curves II refer to fish which were defrosted the first time 48 hours at 18°C.

and III) is greater during defrosting and subsequent storage than in the case of fish frozen in the fresh condition (curve I).

The curves in Figure 3 illustrate the changes in the extractibility of myosin effected by refreezing and subsequent defrosting. Curves I refer to fresh fish that were frozen in the fresh condition (post *rigor mortis*), defrosted by keeping them wrapped in pliofilm in air at 18°C. for 8.5 hours and then refrozen and defrosted. In the other series (curves II) another batch of fresh fish was frozen, defrosted and stored at 18°C. in air for 48 hours, refrozen and defrosted. The extractable myosin decreased rapidly to a low value during second defrosting and subsequent storage. The amount of myosin nitrogen in the brine extract decreases on defrosting and storing after refreezing from 64% of total protein nitrogen to 5.5% during 17 hours at 18°C. (curve I). In the case illustrated by curve II, the myosin nitrogen decreases from 45% to 6.4% during 26 hours in air at 18°C.

DISCUSSION

The extractibility of myosin with salt solution varies greatly with the condition of the fish. During *rigor mortis* the amount of myosin (the term is used in its broader application) present in salt extract is very low, e.g. in the experiment of Figure 1, only 27.6% of total protein nitrogen compared with 68.0% after the resolution of *rigor mortis*. However, the changes undergone by the proteins during *rigor mortis* are reversible. Chilling, freezing and cold storage prolong but do not inhibit the final resolution of *rigor mortis*. During a few days' storage at —20°C., the extractable myosin from *rigor mortis* muscle increases to a higher value. Some observations, however, indicate that freezing during *rigor mortis* and cold storage make myosin more prone to undergo denaturation during defrosting than when the fish are frozen after the resolution of *rigor mortis*. On the other hand, autolytic reactions (the decomposition of protein was experimentally established), or at least conditions which favor these reactions, diminish the tendency of the proteins to undergo denaturation.

Storage of fresh fish at room temperature increases the amount of brine extractable myosin (Figure 2). Storage of frozen fish at room temperature, on the contrary, causes a small loss of myosin extractibility in the beginning; after the frozen fish has been defrosted, a very large loss is noted. Thus the freezing has caused a change in the condition of native proteins, making them more liable to undergo denaturation. Defrosting at 18°C. in air for about 7 hours (incomplete defrosting) causes no considerable loss of myosin solubility, but during continued storage the myosin is denatured rapidly and to a great extent. When the storage time and defrosting temperature is increased, the myosin becomes less and less soluble in salt solution. Refreezing again alters the state of the myosin molecules in such a way that the denaturation increases during defrosting and subsequent storage. These results give no support to the conclusion of Pottinger *et al. (8)*, "that immediate freezing, with subsequent thawing, filleting and refreezing of the fillets causes no marked adverse effect on quality over fillets prepared from freshly-caught iced fish," which view is accepted, e.g. by Hartshorne and Puncochar *(5)*. In fact, it seems true that freezing and refreezing both alter the state of the myosin molecules, making them more sensitive to denaturation during cold storage, defrosting and subsequent storage. This type of denaturation is irreversible in contrast to the alteration of proteins in *rigor mortis*. Furthermore some experimental

observations indicate that the denaturation of fish proteins during freezing and cold storage may differ from the denaturation taking place during defrosting and subsequent storage.

SUMMARY

The deterioration in frozen fish is partly due to a denaturation of the proteins. The extent of the deterioration is largely determined by the condition of the fish when frozen. The least stable of the protein components of fish muscle is the myosin fraction. It has been established that repeated freezing increases the tendency of these proteins to undergo denaturation. This is responsible largely for the deterioration which becomes evident during storage in the frozen condition, during defrosting, and during subsequent storage.

LITERATURE CITED

1. Association of Official Agricultural Chemists. *Methods of Analysis.* Sixth ed., 1945, Washington, D. C.
2. Dyer, W. J. Protein denaturation in frozen and stored fish. *Food Research,* **16**, 522 (1951).
3. Dyer, W. J., French, H. V., and Snow, J. M. Proteins in fish muscle. I. Extraction of protein fractions in fresh fish. *J. Fisheries Research Board Can.,* **7**, 585 (1950).
4. Hamoir, G. The proteins of fish. *Biochem. Soc. Symp.,* No. 6, 8 (1951).
5. Hartshorne, J. C., and Puncochiar, J. F. Freezing fish at sea—New England. Part 1. Preliminary experiments. *Commercial Fisheries Rev.,* **14**, No. 2, 1 (1952).
6. Notevarp, O., and Heen, E. Virkningen av fryschastighet, lagringstemperatur og råstoffets friskhet på kvaliteten av frossen fisk. *Fiskeridirektoratets skrifter. Serie Tekn. Und., 1,* No. 2 (1938).
7. Notevarp, O., and Heen, E. The influence of freezing rate, storage temperature and freshness of raw material on the quality of frozen fish. *Z. ges. Kälte Ind.,* **47**, 122 (1940).
8. Pottinger, S. R., Keer, R. G., and Lanham, W. B. Effect of refreezing on quality of sea trout fillets. *Commercial Fisheries Rev.,* **11**, No. 1, 14 (1949).
9. Reay, G. A. The influence of freezing temperatures on haddock's muscle. Part I. *J. Soc. Chem. Ind.* (London), **52** (T), 265 (1933).
10. Reay, G. A. The influence of freezing temperatures on haddock's muscle. Part II. *J. Soc. Chem. Ind.* (London). **53** (T), 413 (1934).
11. Reay, G. A., and Kuchel, C. C. Proteins of fish. *Rept. Food Inv. Bd., Gr. Brit.* (1936), 93 (1937).
12. Snow, J. M. Proteins in fish muscle. III. Denaturation of myosin by freezing. *J. Fisheries Research Board Can.,* **7**, 599 (1950).

ERRORS OF THE SECOND KIND IN ORGANOLEPTIC DIFFERENCE TESTING

G. A. BAKER, M. A. AMERINE, AND E. B. ROESSLER
University of California at Davis

(Manuscript received September 8, 1953)

An error of the first kind in difference testing by organoleptic methods is committed if it is said that a difference exists when in fact no difference is present. An error of the second kind is made when actual differences are overlooked. Errors of the first kind in organoleptic difference testing have been discussed by several authors *(2, 3, 4, 5, 9, 10, 11)*. In particular, Lockhart *(5)* is quite clear on some points overlooked by others.

The mathematical model for organoleptic difference testing is the binomial distribution. The probability of errors of the second kind based on the binomial distribution is discussed in detail in the literature *(1, 7, 8)*. However, all the published discussions assume an unchanging fundamental probability. Thus, the published results might possibly apply to difference testing by one "competent" judge, but even this is doubtful due to psychological factors operating on the individual judge, which are largely beyond the control of the experimenter. When it comes to the case of panel or general consumer testing the situation is greatly complicated by varying "thresholds," rate of increase of the probability of detecting differences, and direction of preference.

It is the purpose of this paper to present an experiment indicating the importance of varying thresholds and of rates of increase of probability of detecting differences for individuals in panel testing on the probabilities of errors of the second kind. These results will be contrasted with classical results in the literature where it is assumed that all individuals of the panel have identical tasting characteristics. The matter of errors of the second kind are very important because the whole program of flavor improvement is based upon detectable organoleptic differences.

PRELIMINARY THEORETICAL CONSIDERATIONS

Let us first consider a single taster with a constant probability of detecting a difference at a given level. We shall assume (a) that there is a fixed threshold for detecting a difference, and (b) that the probability of detecting a difference is proportional to the concentration present, the concentration being measured on an appropriate scale above the threshold value up to a certain point where the probability of detection becomes and remains one. Thus for this one taster there would be a threshold value, say a_i, and a value for 100% detection, say b_i. The factor of proportionality expressing the relationship between concentration and probability of detection can be designated by k_i, but k_i is expressible in terms of a_i and b_i in the form

$$k_i = \frac{1}{(b_i - a_i)}$$

If we have a series or panel of tasters and denote by a_i, b_i, and k_i the constants characterizing the ith taster, then it is readily seen that the situation is more complex since none of the constants a_i, b_i, and k_i need be even approximately equal.

We shall set the probability of an error of the first kind equal to α and indicate the effect of the situations noted above on the probability of errors of the second kind.

Let us denote the concentration on some scale by x. Then if x is greater than zero in the case of a single taster and equal to or less than a_1, the probability of an error of the second kind is $\frac{1}{2} - \alpha$. For x between a_1 and b_1 the probability of an error of the second kind is a monotone decreasing function which reaches zero at b_1 and remains zero for greater values of x.

In the case of a panel the probability of an error of the second kind will remain $1 - \alpha$ for x greater than zero up to the threshold of the most acute member of the panel and will be greater than zero until the 100% detection point of the worst member has been reached. Thus the curve showing the decrease in the probability of errors of the second kind falls much more slowly and over a much greater range for a panel than for a single taster.

Much the same effect would be produced by a single taster in the case where the a_1, b_1, and k_1 are functions of the trial made.

ILLUSTRATIVE ORGANOLEPTIC EXPERIMENT

To illustrate the ideas developed in the previous section a simple organoleptic experiment was carried out. Concentrations of sucrose in distilled water were prepared as follows:

Number of sucrose solution	Concentration of sucrose in percent
0.5	0.017
1	0.034
2	0.068
3	0.137
4	0.274
5	0.548

A simple paired test procedure was used. The taster was asked which of two solutions — one distilled water and the other containing a sucrose solution — is the sweeter. The tasters consisted of the three authors, members of a class in enology of one of the authors, and research students in enology. The experiment was conducted during the second semester 1952-53. Five trials were made on each tasting day by each taster until at least 15 trials were made on every concentration except for the solution 0.5 where it was obvious that only one taster had any hope of detecting a difference. The pairs were presented in an approximately random order and so that several concentration solutions were tasted each time. The observed data are presented in Table 1. In considering Table 1 it should be remembered that the number of trials for each solution is not large and that the binomial variance is largest for the probability of success equal to one-half and becomes zero as the probability of detection approaches one.

The ratios of right choices to total choices for each of the tasters in Table 1 were computed and plotted. A study of these graphs strongly suggests the following hypotheses:

1. The probability of detecting a difference after a certain concentration (a_1) to the concentration of 100% detection (b_1) is at least approximately linear in terms of the scale chosen. This conforms to the Fechner-Weber relation (6).
2. All but one detected 100% at the level of the 5th concentration. The one exception showed 14 right choices out of 15.
3. The thresholds (a_1) varied from level 0.5 to level 4. It follows that the k_1 are quite different for the different tasters.
4. The tasters could be grouped at least roughly. Group I (A, C, F, I) had a threshold at 3 or above and a 100% detection value of 4 or less. Thus the slope of the probability of detection line (k_1) was large. Group II (K, L, O) had a threshold of 2 or less and a 100% detection point of 4 or less. The k_1 are about half those for Group I. Group III consists of one taster (J) whose threshold is 0.5 or less and 100% detection point 4 or less. His k_1 is comparatively small. Group IV (G, H) is very similar to Group I except that the

TABLE 1

Paired difference tests on six sucrose solutions for 15 tasters in 1953

Taster	Solutions											
	0.5		1		2		3		4		5	
	Right	Wrong	Right	Wrong	Right	Wrong	Right	Wrong	Right	Wrong	Right	Wrong
A	....	....	11	8	12	5	10	9	15	0	15	0
B	....	....	8	11	7	10	8	11	12	3	15	0
C	....	....	8	11	10	8	12	6	15	0	15	0
D	....	....	10	9	9	9	12	6	13	2	15	0
E	....	....	12	5	7	12	10	9	13	2	15	0
F	....	....	6	13	11	6	11	8	15	0	15	0
G	....	....	13	6	11	6	11	8	9	6	15	0
H	....	....	11	8	10	7	9	10	9	6	15	0
I	....	....	8	9	8	11	7	12	15	0	15	0
J	8	8	22	9	15	4	16	3	15	0	15	0
K	....	....	10	9	11	6	14	5	15	0	15	0
L	....	....	9	8	9	10	13	6	14	1	14	1
M	....	....	9	10	12	6	16	2	11	4	15	0
N	....	....	10	9	13	4	16	3	14	1	15	0
O	....	....	7	12	12	5	14	5	15	0	15	0

threshold is 4 approximately and the 100% detection point is 5 or less. Group V (B, E) is very similar to Group II except that the threshold and 100% detection points are moved up about one unit. Group VI (D, M, N) show a threshold of about 1 and a 100% detection point of about 5. The improvement in ability to detect differences for this group shows a rather slow increase with increase of concentration.

5. There is a linear relationship between concentration and the number of tasters detecting a difference at a given concentration measured on our present scale. Inspection of the graphs for the 15 tasters gives the following results:

Concentration number	No detecting differences	
	Observed	Expected
0.5	0	0
1	1	1.24
2	5	4.67
3	8	8.10
4	13	11.53
5	15	14.96

The theoretical or expected ordinates are obtained by assuming a straight line having a concentration-intercept of $\frac{5}{8}$ and a slope of 3.43.

PROBABILITIES OF ERRORS OF THE SECOND KIND

The experiment discussed in the previous section supports very strongly the general ideas discussed earlier. In particular, the experiment indicates that the power or logarithmic scale is the proper scale for measuring concentration to achieve linearity of increase of detectability with increase of concentration. Also the thresholds, a_i, vary from approximately 0.5 to 4 on this scale and the 100% detection points, b_i, vary from 4 to 5. The k_i also vary markedly as some tasters show a very gradual improvement in detectability while others show a very rapid increase.

The data of Table 1 really give the number of right choices and number of total choices. Of course, when the ratio of right choices to total choices is 0.5 the probability of detection of an existing difference is zero. When the ratio is one, then the probability of detection of an existing difference is one or certainty.

For purposes of illustration take α (probability of error of the first kind) equal to 0.05 and consider that n (total number of choices) is 10. Then the critical region is the number of terms of $\left(\frac{1}{2}+\frac{1}{2}\right)^{10}$ on the upper end necessary to give a probability of 0.05 which is approximately given by the last three terms. Suppose we consider the situation for Group I where the threshold value is 3 and the 100% detection point is 4. The probabilites of errors of the second kind will behave as follows:

Concentration	Probability of right choice	Probability of error of second kind
>0 and ≤3	0.50	0.95
3.20	0.60	0.83
3.40	0.70	0.62
3.60	0.80	0.32
3.80	0.90	0.07
4.00 and greater	1.00	0.00

The probabilities of errors of the second kind for all groups and for the total group (n = 10) are compared in Table 2. The observed curves are sight smoothed using straight lines for the homogeneous groups, but a smooth curve was used for the combination of the groups.

For other values of n and α the relationships portrayed in Table 2 would remain relatively the same.

TABLE 2

Probabilities of errors of the second kind, n = 10, α = 0.05, for six groups and their combinations *

Concentration	Probabilities of errors of the second kind						
	I	II	III	IV	V	VI	Combination
0.5	.95	.95	.95	.95	.95	.95	.95
1.0	.95	.95	.83	.95	.95	.95	.93
2.0	.95	.83	.47	.95	.95	.74	.85
3.0	.95	.32	.07	.95	.95	.32	.69
4.0	.00	.00	.00	.95	.32	.07	.07
5.0	.00	.00	.00	.00	.00	.00	.00

* The values of this table are readily obtained from "Tables of the Binomial Probability Distribution," National Bureau of Standards, Applied Mathematics Series 6, 1950.

Since the probabilities of errors of the first kind remain the same for all groups we see that Group III consisting of one taster is far superior to any other. Group IV is the worst. The combination of all tasters gives a slow, prolonged, and non-uniform improvement in probabilities of errors of the second kind.

SUMMARY

A simple organoleptic experiment on the detection of sucrose in distilled water by means of paired tests has been presented. At the present level of experimentation it appears that the probability of making the right choice out of a pair increases linearly with respect to the logarithm of the concentration but that these linear relationships varied greatly for different tasters with somewhat similar training and background. Certain possible groupings are indicated. Also, these data indicate that the number of tasters detecting a given concentration may be linear on a log-concentration scale.

From the standpoint of detecting organoleptic differences if they exist it is important to use the best taster available. The probabilities of errors of the second kind vary greatly from taster-to-taster and from group-to-group. A combination of tasters gives results that are much poorer than the best but somewhat better than the worst.

Present experimental methods control errors of the first kind satisfactorily, but without proper attention to errors of the second kind progress will be slow and its cost will greatly exceed what is necessary to achieve the desired results.

LITERATURE CITED

1. Anderson, R. L., and Bancroft, T. A. *Statistical Theory in Research.* 1952, McGraw-Hill Book Co., New York, N. Y.
2. Boggs, M. M., and Hanson, H. L. Analysis of foods by sensory difference tests. *Recent Advances in Food Research*, **2**, 219-258 (1949).
3. Harrison, S., and Elder, L. W. Some applications of statistics to laboratory taste testing. *Food Technology*, **4**, 434-439 (1950).
4. Highman, Arthur. How many samples make a valid test? *Western Industry*, May, 1952.
5. Lockhart, Ernest E. Binomial systems and organoleptic analysis. *Food Technology*, **5**, 428-431 (1951).
6. Moncrieff, R. W. *The Chemical Senses.* 1944, John Wiley and Sons, New York, N. Y., pp. 1-424, especially p. 54.
7. Mood, A. M. *Introduction to the Theory of Statistics.* 1950, McGraw-Hill Book Co., New York, N. Y.
8. Neyman, J. First Course in Probability and Statistics. 1950, Henry Holt and Co., New York, N. Y.
9. Peryam, David R., and Swartz, VeNona W. Measurement of sensory differences. *Food Technology*, **4**, 390-395 (1950).
10. Roessler, E. B., Warren, J., and Guymon, J. F. Significance in triangular taste tests. *Food Research*, **13**, 503-505 (1948).
11. Wood, Eric C. Organoleptic tests in the food industry. III. Some statistical considerations in organoleptic tests. *Journal of the Society of Chemical Industry*, **68**, 128-131 (1949).

THE LEAD, ZINC AND COPPER CONTENT OF FOODS

DORIS LARKIN, MARGARET PAGE, J. C. BARTLET, AND ROSS A. CHAPMAN
Food and Drug Laboratories, Ottawa, Canada

(Manuscript received September 12, 1953)

In considering the lead, zinc and copper content of foods, one must keep in mind that while the presence of these inorganic elements may be due to contamination, zinc and copper in particular also occur naturally in many products. Parsons *(8)* has summarized data given in the literature for naturally-occurring lead, zinc and copper in foods. Examples of relatively high values found without any apparent extraneous contamination are shown in Table 1.

TABLE 1
Lead, zinc and copper naturally present in foods (8)

Element	Material	p.p.m.
Lead	Oysters	80-100*
	Bones	15-140
Zinc	Wholemeal flour	20-50
	Oatmeal	140
	Beef liver	30-85
	Pig liver	30-150
	Herrings	700-1200*
	Oysters	2000*
Copper	Butter beans	12
	Indian tea	16
	Ox liver	20-80
	Calf liver	150
	Lobster	167
	Oysters	1600*

*Probably grown in contaminated water.

It is of interest to note from these data that cereals are relatively high in zinc; copper and zinc tend to accumulate in the liver while lead is found most frequently in the bones. Shellfish, crustacea and fish can accumulate quite appreciable amounts of these elements if grown in contaminated waters. This is not a condition generally encountered as Dick and Pugsley *(2)* have reported that in samples of canned shellfish and crustaceans obtained on the Canadian market the content of copper and lead was relatively low. However, one sample of oysters contained 65 p.p.m. of copper and one can of lobster was found with a lead content of 13 p.p.m.

More important, however, than the naturally-occurring inorganic elements from the health point of view, is the possibility of adventitious or deliberate addition. Lead may find its way into foods from insecticide sprays and from lead piping or other food processing equipment. Occasional examples of contamination with lead from the solder of tin containers are still encountered, but in most modern cans no solder comes in contact with the food. The use of lead foil in wrapping foods has largely

been abandoned, but at one time this procedure resulted in appreciable contamination of confectionery, cheese and tea packed in chests lined with lead foil.

The most important source of contamination of lead in fruits and vegetables is from spray residues. This possibility has been reduced in recent years by the use of organic insecticides but large amounts of lead arsenate are still employed for this purpose. Wichmann *(11)* has reported residues of lead on apples as high as 8 p.p.m. Tea in lead-lined chests has been found to contain up to 25 p.p.m. of lead by Forstner and desiccated coconut in similar chests contained 45 p.p.m. in the surface layer *(3)*. Forstner *(3)* also reports the contamination of canned beef and luncheon meat packed in tin containers in which the meat had contact with solder around a "stuffing hole" or along a longitudinal seam. He obtained values as high as 200 p.p.m. of lead in the slice of corned beef immediately adjacent to the solder. In luncheon meat the values were not as high but 13 samples contained from 20 to 30 p.p.m. of lead. In this laboratory samples of canned pork containing 7 to 15 p.p.m. of lead in the portion adjacent to the seam have been encountered in recent years.

Zinc occurs naturally in a wide variety of food products and is present in appreciable amounts in cereals, eggs, meats and such vegetables as beans and peas. Waldor *(10)* has estimated that the normal intake of zinc per day by an average adult is approximately 15 mg. Sylvester and Hughes *(9)* found amounts of zinc as high as 7.4 p.p.m. in beet root, 7.1 p.p.m. in spinach, 29 p.p.m. in egg yolk, 54 p.p.m. in cocoa nibs, 42 p.p.m. in tea, 112 p.p.m. in wheat bran and 145 p.p.m. in wheat germ. As Forstner *(3)* has pointed out, the use of galvanized vessels has been the major source of contamination of foods with zinc, but with the advent of modern processing equipment such contamination is no longer a major problem. However, in this laboratory samples of gelatin and agar have been examined recently which contained 125 and 750 p.p.m. of zinc respectively. These products had apparently been contaminated by drying on galvanized trays or wire netting.

Copper is present in most foods in relatively small amounts, usually not exceeding 2 p.p.m. However, McCance and Widdowson *(5)* report values for copper as high as 13.9 p.p.m. in dried skim milk, 58 p.p.m. in liver, 34 p.p.m. in cocoa, 15.9 p.p.m. in tea, 11.1 p.p.m. in chocolate, 12.2 p.p.m. in butter beans and 11 p.p.m. in Brazil nuts. Some copper may appear in processed foods from worn equipment and also from fungicidal sprays used on plants.

Parsons *(8)* has attempted to classify inorganic contaminants of foods on the basis of their effect on the human system. In such a classification lead falls into the group which is harmful and non-essential while copper and zinc are placed with those which are essential but harmful in excess. There is little difference of opinion regarding metals such as lead. This element is definitely harmful and therefore its presence in food should be reduced to the lowest possible minimum. In case of zinc and copper, both are essential elements and as such are necessary in the human diet. In regard to the possible harmful effects of these elements if ingested in excessive amounts, Parsons *(8)* points out that all that can be said with

certainty is that in moderately large doses, the salts of inorganic acids do have emetic properties. Therefore, while small amounts of zinc and copper are desirable the presence of excessive amounts of these elements in foods is discouraged.

The most recent comprehensive surveys of the lead content of foods were conducted by Monier-Williams in 1938 *(7)* and by Kehoe, Cholak and Story in 1940 *(4)*. However during the past decade there has been a marked change in the insecticide sprays employed on food products with a definite trend toward the newer organic insecticides. In addition, there have been marked changes in processing techniques and an increase in the use of stainless steel throughout the food industry. In view of these developments it was considered desirable to conduct a survey of the lead, zinc and copper content of foods produced under modern conditions. Emphasis has been placed on the examination of processed foods which might have been exposed to metallic contamination. No attempt was made to determine extremely small quantities of copper and zinc which might be of interest to the nutritionist. Surveys have recently been conducted in this laboratory on the heavy metal content of canned shellfish and crustaceans *(2)* and gelling agents *(1)*. Therefore these classes of products have not been included in the present survey.

ANALYTICAL METHODS

Lead, zinc and copper were determined spectrographically by the method described by Bartlet *et al. (1)*.

A weighed sample was ashed with nitric, sulphuric and perchloric acids. To an aliquot of the sample, 2 mg. of bismuth was added and the heavy metals were precipitated as sulphides with the bismuth acting as carrier. The sulphides were mixed with graphite powder and the metals determined spectrographically, employing a d-c arc at 6 amp. The densities of lines of bismuth, lead, zinc and copper were measured with a microphotometer; intensity ratios of impurity lines to a bismuth line were calculated and the amount of each element was determined from standard curves.

The results are all based on single determinations except where high results were obtained. Analysis was repeated when the lead found was greater than 2 p.p.m. in solid food or 0.2 p.p.m. in liquids and when the zinc and copper exceeded 50 p.p.m.

RESULTS AND DISCUSSION

A total of 171 food samples were analyzed in this investigation. Only one value has been reported where similar results were obtained on the same type of product. The results are shown in Tables 2 to 9.

The sample of canned luncheon meat No. 1 reported in Table 2 as containing 4 p.p.m. of lead was probably contaminated from the can since the container was considerably etched and the sample included some surface material. The value of 60 p.p.m. of zinc in beef purée may have resulted from the addition of liver to this product although Mitteldorf and Landon *(6)* have recently reported values as high as 53 p.p.m. of zinc in beef rib eye samples.

The values obtained on the flavoring preparations shown in Table 3 were satisfactory but two of the samples of the beverage powders contained 2.7 and 3.3 p.p.m. of lead. These amounts of lead are considered excessive. The source of the lead in these products was not ascertained. The metallic content of the dessert powders, cake mixes and cereal prod-

TABLE 2

Meat and dairy products, lard and shortening

	Lead p.p.m.	Zinc p.p.m.	Copper p.p.m.
Liver purée	1.1	55	<5
Beef purée	0.8	60	<5
Beef and pork luncheon meat			
1	4.0	38	<5
2	1.5	32	<5
3	0.6	32	<5
4	0.9	32	<5
5	0.8	33	<5
Beef luncheon loaf	1.0	32	<5
Beef and vegetable dinner	1.3	28	<5
Milk, powdered skim			
1	0.6	44	<5
2	1.3	47	<5
Eggs, powdered whole	<0.5	55	5
Lard			
1	<0.5	<5	<5
2	0.7	<5	<5
3	<0.5	<5	<5
Shortening			
1	0.6	6	<5
2	1.3	<5	<5
3	<0.5	7	<5
4	<0.5	<5	<5

TABLE 3

Flavoring preparations and beverage powders

	Lead p.p.m.	Zinc p.p.m.	Copper p.p.m.
Butterscotch, artificial	0.05	1	<5
Strawberry, artificial	0.05	2	<5
Maple, artificial	0.11	1.5	<5
Rum, artificial	0.05	<1	<5
Coconut, artificial	0.04	<1	<5
Lemon, artificial	0.04	<1	<5
Peppermint, true	0.18	<1	<5
Lemon, true	0.09	<1	<5
Vanilla, true	0.11	<1	<5
Orange, true	0.04	<1	<5
Lime beverage powder	2.7	<5	<5
Lemon beverage powder	3.3	<5	<5
Strawberry beverage powder	0.9	<5	<5

uets as shown in Tables 4 and 5 was considered satisfactory with the exception of the orange gelatin dessert and the oatmeal mixture No. 1 which contained 77 and 60 p.p.m. of zinc respectively. The excessive zinc in the dessert powder was probably largely contributed by the gelatin as Bartlet *et al. (1)* have reported values up to 125 p.p.m. of zinc in pure gelatin.

The values given in Table 6 indicate a relatively high level of lead and zinc in a number of the spices and the amount of lead in the curry

powders Nos. 1 and 2 is definitely excessive. However the values for lead in both curry powders and turmeric are much lower than those previously reported by Monier-Williams *(7)* and by Forstner *(3)*. In these earlier reports contamination was confirmed but the source of the lead in the samples examined in this investigation is not known. No explanation was found for the high values for zinc obtained on a number of the spices.

The results obtained on canned fruit products, Table 8, were satisfactory with the possible exception of one sample of raspberry jam with added pectin which contained 2.6 p.p.m. of lead. However, there was no evidence that appreciable amounts of spray residues were carried over into

TABLE 4

Dessert powders and cake mixes

	Lead p.p.m.	Zinc p.p.m.	Copper p.p.m.
Cherry gelatin dessert powder	<0.5	14	<5
Raspberry gelatin dessert powder	1.1	11	<5
Cherry gelatin dessert powder	1.5	<5	<5
Orange gelatin dessert powder	2.0	77	<5
Pineapple gelatin dessert powder	1.9	<5	<5
Chocolate pudding mix	<0.5	14	<5
Tapioca dessert mix	<0.5	<5	<5
Caramel dessert mix	<0.5	7	<5
Lemon pie filling	<0.5	<5	<5
Chocolate frozen custard	0.6	26	<5
Chocolate ice cream mix	0.5	31	<5
White cake mix	0.5	8	<5
Gold cake mix	<0.5	11	<5
Chocolate cake mix			
1	0.5	11	<5
2	<0.5	17	5
Cookie mix	0.9	17	<5

TABLE 5

Cereal products

	Lead p.p.m.	Zinc p.p.m.	Copper p.p.m.
Flour, all purpose	0.8	<5	<5
Bread, whole wheat			
1	1.1	24	<5
2	1.2	35	<5
Bread, white	0.5	<5	<5
Bread, rye	1.1	22	<5
Corn flakes	<0.5	7	<5
Rice cereal	<0.5	19	<5
Bran cereal	<0.5	<5	9
Bran cereal with raisins	0.6	<5	13
Infants' cereal food			
1	2.4	43	<5
2	2.1	24	<5
Infants' oatmeal mixture			
1	1.5	60	<5
2	2.2	31	<5
Infants' mixed cereal	1.9	37	<5

TABLE 6

Spices, condiments and dressings

	Lead p.p.m.	Zinc p.p.m.	Copper p.p.m.
Allspice	4.0	5	<5
Curry powder			
1	17.0	77	<5
2	14.3	85	25
3	4.3	33	<5
Cinnamon	4.5	31	<5
Ginger	4.2	55	<5
Mace	1.4	33	<5
Nutmeg	0.5	31	<5
Pepper, black	0.9	26	<5
Pepper, cayenne	2.9	43	<5
Pepper, white	0.6	24	<5
Pastry spice	6.0	49	<5
Pickling spice	8.0	54	<5
Sausage seasoning	1.0	<5	<5
Turmeric			
1	2.4	30	<5
2	6.8	72	<5
3	2.9	47	<5
4	2.2	57	24
Sweet relish	0.6	<5	<5
Mustard with horseradish	<0.5	24	<5
Mustard, prepared	<0.5	21	<5
Horseradish	<0.5	16	<5
Worcestershire sauce	<0.5	13	<5
Meat sauce	1.2	<5	<5
Catsup			
1	0.6	17	<5
2	<0.5	<5	<5
Salad dressing	0.6	<5	<5
French dressing	<0.5	<5	<5
Salt	0.6	<5	<5

TABLE 7

Canned fruits, fruit juices, jams and jellies

	Lead p.p.m.	Zinc p.p.m.	Copper p.p.m.
Cherries			
1	1.8	16	<5
2	1.5	22	<5
Pears			
1	0.9	14.5	<5
2	0.7	17.5	<5
Plums			
1	1.1	17	<5
2	1.1	<5	<5
Apple sauce			
1	1.0	12	<5
2	0.9	<5	<5
3	0.7	13.5	<5
Apple juice			
1	0.06	1.9	<5
2	0.11	<1	<5
3	0.07	<1	<5
Jam, strawberry, added pectin and color	<0.5	<5	<5
Jam, raspberry, added pectin and color	2.6	<5	<5
Jam, pure raspberry	0.8	<5	<5
Jelly, pure grape	<0.5	<5	<5
Jelly, pure apple	0.5	<5	<5

TABLE 8
Beverages

	Lead p.p.m.	Zinc p.p.m.	Copper p.p.m.
Carbonated beverage			
1	<0.01	<1	<5
2	0.02	<1	<5
3	0.03	<1	<5
4	0.04	<1	<5
5	0.08	<1	<5
6	0.1	<1	<5
7	0.04	1.5	<5
8	0.03	3.2	<5
9	0.02	1.4	<5
10	0.12	<1	<5
Chocolate drink	0.03	4.6	<5
Concentrated collins mixer	0.09	<1	<5
Dairy malt, powder	1.2	26	<5
Grape juice, concentrated	0.02	<1	<5
Reconstituted lemon juice	1.1	<1	<5
Tea, dried leaves			
1	1.1	<5	<5
2	1.6	<5	<5
Tap water	0.04	<1	<5

TABLE 9
Miscellaneous

	Lead p.p.m.	Zinc p.p.m.	Copper p.p.m.
Baking soda	<0.5	<5	<5
Baking powder	<0.5	<5	<5
Cocoa			
1	2.8	<5	40
2	2.0	102	46
Cream of tartar	2.5	<5	<5
Dehydrated potato	2.1	39	<5
Dried hops	9.3	90	<5
Malt extract	<0.5	<5	<5
Yeast, dried	1.4	80	50
Sugar, brown			
1	0.5	<5	<5
2	1.2	<5	<5
Sugar, white granulated			
1	1.2	<5	<5
2	0.9	<5	<5
Table syrup	0.7	21	<5
Syrup	<0.5	<5	<5
Maple syrup			
1	1.3	105	<5
2	1.5	52	<5

the processed products. The lead and zinc content of the beverages shown in Table 8 was considered satisfactory. Of the miscellaneous products listed in Table 9, cocoa, dried hops, yeast and maple syrup contained from 80 to 105 p.p.m. of zinc. The maple syrup was probably contaminated by use of galvanized equipment employed in the collection or processing of

this material. The source of the zinc in the other products is less apparent. It is known that yeast tends to concentrate metallic ions from the medium in which it is grown and the zinc naturally present in the hops would be concentrated by dehydration. It would appear that the cocoa had been contaminated during processing.

SUMMARY

A total of 171 Canadian processed foods have been analyzed for their lead, zinc and copper content. The results indicate that in general the lead, zinc and copper content of the processed foods examined is satisfactory.

LITERATURE CITED

1. Bartlet, J. C., List, Ethel, Page, Margaret, and Chapman, R. A. The heavy metal content of gelling and stabilizing agents. *Can. J. Tech.*, **31**, 146 (1953).
2. Dick, J., and Pugsley, L. I. The arsenic, lead, tin, copper, and iron content of canned clams, oysters, crabs, lobsters and shrimps. *Can. J. Res.*, **28F**, 199 (1950).
3. Forstner, G. E. The occurrence of metallic contaminants in foods. *Chem. Ind.*, 499 (1948).
4. Kehoe, R. A., Cholak, J., and Story, R. V. A spectrochemical study of the normal ranges of concentration of certain trace metals in biological materials. *J. Nutrition*, **19**, 579 (1940).
5. McCance, R. A., and Widdowson, E. M. *The Chemical Composition of Foods.* 1946, H. M. Stationery Office, London.
6. Mitteldorf, Arthur J., and Landon, Donald O. Spectrochemical determination of the mineral-element content of beef. *Anal. Chem.*, **24**, (3,469) (1952).
7. Monier-Williams, G. W. Lead in foods. Reports on Public Health and Med. Subj., No. 88. H. M. Stationery Office, London (1938).
8. Parsons, A. T. Problems arising from the use of chemicals in food. *Chem. Ind.*, **18**, 384 (1952).
9. Sylvester, N. D., and Hughes, E. B. The determination of zinc in foods. *Analyst*, **61**, 734 (1936).
10. Waldor, Winnifred O. Food poisoning by metallic salts. *Food*, **20**, 96 (1951).
11. Wichmann, H. J. Report on metals in foods. *J. Assoc. Off. Agr. Chemists*, **18**, 182 (1935).

AMINO ACID COMPOSITION OF ORGAN MEATS[a]

B. S. SCHWEIGERT, BARBARA A. BENNETT, AND BARBARA T. GUTHNECK

Division of Biochemistry and Nutrition, American Meat Institute Foundation and Department of Biochemistry, The University of Chicago, Chicago, Illinois

(Manuscript received September 12, 1953)

During the past several years studies have been conducted to determine the amino acid composition of fresh and cooked pork, beef, and lamb muscle cuts and processed meat items (sausages, canned meats, etc.) *(5, 9-11)*. These studies have now been extended to pork, beef, and lamb organ meats. Data on samples of certain of the organ meats are already available *(1-4, 6, 7, 12)*. In the present study the amounts of leucine, valine, isoleucine, threonine, phenylalanine, methionine, arginine, histidine, lysine, and tryptophan in pork, beef, and lamb liver, kidney, heart, brain, spleen, lung, and pancreas have been determined.

EXPERIMENTAL AND RESULTS

Samples of organ meats were obtained from 2 meat packing companies, each sample was a composite of organs taken from 10 different animals. Additional composite samples of pork liver were obtained from old sows (325 pounds in weight) and of beef liver from old cows (cutters and canners). A detailed description of the sampling technique, proximate composition, and thiamine, riboflavin, and niacin composition of these samples has been published previously *(8)*.

Analyses for all amino acids except tryptophan were made microbiologically by methods previously described by this laboratory. Tryptophan was determined chemically by methods we also have used previously. The analyses were made on the dried samples and the amino acid composition of the protein and of the fresh sample were calculated from these data and the proximate analyses. All samples were analyzed at least in duplicate.

The data for the amino acid composition of the beef organs are presented in Table 1, for pork organs in Table 2, and for lamb organs in Table 3. The percentage of crude protein ($N \times 6.25$) in the undried samples is also included. From the amino acid and protein data presented, the percentage of each amino acid in the undried sample can be readily calculated.

For the organ meats studied, leucine, valine, methionine, and possibly threonine were higher in liver protein and several of the amino acids were lower in spleen and lung protein. The tryptophan content of different organs varied the most. It is of particular interest that the concentration of tryptophan in the crude protein of pancreas (beef and pork) was high. This was the most striking for the pork pancreas samples (Table 2). Certain enzyme preparations from pancreas were available and these were analyzed for tryptophan. The percentage of tryptophan in the protein of pancreatin, a crude trypsin preparation, and of crystalline trypsin was high also (2.15, 2.33, and 1.94%, respectively).

The results obtained for 12 samples of pork and 12 lamb muscle cuts, 24 beef muscle cuts and 26 processed meats, as well as those from the present study on pork, beef, and lamb organ meats have been summarized in Table 4.

From an overall evaluation, the amino acid composition of the crude protein of these samples is similar for muscle and organ meats. Certain differences will be noted, however. The organ meats tend to be higher in the percentage of leucine, valine, and phenylalanine, and lower in methionine, arginine, and histidine in the protein than in muscle cuts or processed meats.

---

[a]Journal Paper No. 78, American Meat Institute Foundation.

TABLE 1

Amino acid composition of beef organs
(All amino acid data expressed as the percentage in the crude protein)

Sample	Protein[b]	Leucine	Valine	Isoleucine	Methionine	Phenylalanine	Threonine	Arginine	Histidine	Lysine	Tryptophan
Liver (1)[c]	19.0	11.8	6.7	6.3	2.57	5.7	5.3	6.2	2.8	9.1	1.54
Liver (2)	20.0	10.3	6.6	5.5	2.42	5.1	5.3	5.8	2.8	9.2	1.55
Liver—Cow (1)	20.9	8.7	6.9	5.7	2.27	5.4	4.6	5.4	2.8	8.7	1.58
Liver—Cow (2)	19.5	10.3	6.3	5.2	2.27	5.0	4.7	5.6	2.7	9.0	1.45
Kidney (1)	16.9	9.0	5.6	4.5	2.25	5.1	4.5	6.6	2.4	8.0	1.43
Kidney (2)	15.7	9.1	5.8	4.5	1.90	4.6	4.3	6.2	2.4	7.9	1.32
Heart (1)	15.5	8.7	5.5	5.1	2.05	4.8	4.4	6.5	2.3	8.7	1.15
Heart (2)	16.6	9.6	5.6	5.1	2.25	4.6	4.9	6.3	2.6	9.2	1.17
Brain (1)	11.0	9.5	6.0	5.3	2.13	5.3	4.7	6.5	3.1	8.4	1.58
Brain (2)	10.9	9.1	5.4	5.4	2.12	5.4	5.2	5.9	2.8	8.3	1.33
Pancreas (1)	12.9	8.2	5.6	5.7	1.84	4.1	4.7	5.8	2.1	8.1	1.62
Pancreas (2)	15.1	8.5	5.7	5.0	1.92	4.6	5.0	5.3	2.2	7.6	1.54
Lung (1)	17.8	8.4	6.0	4.2	2.00	4.6	4.1	6.1	2.4	7.5	1.00
Lung (2)	18.8	8.1	5.7	4.6	1.62	4.3	4.0	6.1	2.2	7.8	0.97
Spleen (1)	16.9	8.0	6.2	4.6	2.11	4.5	4.6	5.9	2.4	7.7	1.08
Spleen (2)	18.4	9.0	5.8	4.5	1.87	4.4	4.1	5.4	2.4	7.9	1.13

[b] % in the fresh (undried) sample.
[c] The numbers in parentheses refer to the source (Company No. 1 and 2) of the organs used in preparing the composite samples.

TABLE 2

Amino acid composition of pork organs
(All amino acid data expressed as the percentage in the crude protein)

Sample	Protein[a]	Leucine	Valine	Isoleucine	Methionine	Phenylalanine	Threonine	Arginine	Histidine	Lysine	Tryptophan
Liver (1)[b]	20.5	9.2	6.9	6.4	2.46	5.5	4.9	5.9	2.8	8.8	1.45
Liver (2)	20.5	10.0	6.7	5.5	2.34	5.0	4.7	5.8	2.9	8.2	1.39
Liver—Sow (1)	21.8	9.3	6.1	5.5	2.12	5.3	4.4	5.1	2.7	8.4	1.42
Liver—Sow (2)	18.9	10.1	6.3	6.5	2.32	5.3	4.8	6.3	2.9	9.4	1.38
Kidney (1)	15.1	9.1	6.5	6.2	1.95	5.2	4.6	5.9	2.4	7.6	1.50
Kidney (2)	16.0	8.9	5.7	5.2	2.26	4.6	4.4	5.8	2.8	7.9	1.32
Heart (1)	16.4	8.9	5.4	6.0	2.19	4.7	4.6	5.6	2.4	8.3	1.19
Heart (2)	16.8	9.5	5.7	5.4	2.30	4.6	4.5	6.2	2.6	8.7	1.23
Brain (1)	11.4	8.6	6.2	5.3	1.95	5.1	4.8	5.4	2.6	8.3	1.55
Brain (2)	11.2	8.9	6.0	5.3	2.05	5.2	5.2	5.2	2.7	9.0	1.34
Pancreas (1)	17.3	7.9	5.9	5.8	1.78	5.0	4.6	5.7	2.2	7.3	2.34
Pancreas (2)	16.4	8.2	5.5	5.2	1.48	4.6	4.2	5.3	2.4	7.6	2.06
Lung (1)	14.0	7.6	5.9	4.8	1.73	4.0	3.9	4.9	2.3	7.8	0.94
Lung (2)	11.7	7.8	6.3	4.3	1.85	4.3	3.8	5.7	2.4	7.5	0.86
Spleen (1)	16.2	8.1	6.1	5.1	2.10	4.7	4.4	5.6	2.3	8.2	1.03
Spleen (2)	17.3	9.3	5.6	5.6	1.95	4.5	4.2	5.5	2.7	7.9	1.15

[a] % in the fresh (undried) sample.
[b] The numbers in parentheses refer to the source (Company No. 1 and 2) of the organs used in preparing the composite samples.

TABLE 3

Amino acid composition of lamb organs
(All amino acid data expressed as the percentage in the crude protein)

Sample[g]	Protein[f]	Leucine	Valine	Isoleucine	Methionine	Phenylalanine	Threonine	Arginine	Histidine	Lysine	Tryptophan
Liver (2)	20.6	10.0	6.2	5.8	2.35	5.8	5.2	6.8	2.9	8.8	1.64
Kidney (2)	15.6	9.0	6.0	5.7	1.97	4.4	4.7	6.0	2.6	8.4	1.04
Heart (2)	14.3	9.1	5.5	5.0	2.33	4.7	4.5	6.5	2.5	8.5	1.15
Brain (2)	11.8	9.2	5.5	5.2	2.53	5.3	4.8	5.6	2.8	9.3	1.42
Pancreas (2)	15.9	8.3	5.1	4.4	1.78	4.3	4.1	5.1	2.3	8.2	1.25
Lung (2)	17.9	9.1	5.7	4.8	1.95	5.0	4.8	6.2	2.5	7.4	0.90
Spleen (2)	18.6	9.4	5.7	5.2	1.93	4.9	5.0	5.8	2.7	8.7	1.38

[f] % in the fresh (undried) sample.
[g] The numbers in parentheses refer to the source (Company No. 1 and 2) of the organs used in preparing the composite samples.

TABLE 4

Comparison of amino acid composition of pork, beef and lamb muscle and organ meats and processed meats[h]
(All values expressed as the percentage in the crude protein)

Sample	Leucine	Valine	Isoleucine	Methionine	Phenylalanine	Threonine	Arginine	Histidine	Lysine	Tryptophan
Muscle Cuts										
Beef	8.4	5.7	5.1	2.3	4.0	4.0	6.6	2.9	8.4	1.10
Pork	7.5	5.0	4.9	2.5	4.1	5.1	6.4	3.2	7.8	1.35
Lamb	7.4	5.0	4.8	2.3	3.9	4.9	6.9	2.7	7.7	1.32
Organ Meats										
Beef	9.1	6.0	5.1	2.1	4.8	4.7	6.0	2.5	8.3	1.34
Pork	8.8	6.1	5.5	2.1	4.9	4.5	5.6	2.6	8.2	1.38
Lamb	9.2	5.7	5.2	2.1	4.9	4.7	6.0	2.6	8.4	1.25
Processed Meats	7.4	5.2	4.9	2.2	4.0	3.9	6.6	2.8	7.4	1.04

[h] Data for muscle and processed meats are taken from previous publications (5, 9-11).

Data obtained in the present study on the amino acid composition of organ meats are in good agreement with those reported in the literature.

SUMMARY

The amounts of leucine, valine, isoleucine, threonine, phenylalanine, methionine, tryptophan, arginine, histidine, and lysine were determined in composite samples of liver, kidney, heart, brain, pancreas, lung, and spleen from beef, pork, and lamb, respectively. On an overall basis the amino acid composition was similar for all organs and was in the same range as observed for muscle cuts studied previously in our laboratory.

Certain differences were observed, however. On evaluations based on the amino acid composition of the protein, liver was shown to be higher in leucine, valine, methionine, and possibly threonine than were other organs; lung and spleen protein tended to be lower, compared with other organs, in several of the amino acids.

The tryptophan content of the protein of pork pancreas was considerably higher than for the other organs studied. Further studies with enzyme preparations from pancreas (pancreatin and trypsin) showed that the protein of these samples also was high in tryptophan content.

LITERATURE CITED

1. Block, R. J., and Bolling, D. *The Amino Acid Composition of Proteins and Foods.* 1951, Charles C. Thomas, Springfield, Illinois.
2. Greenhut, I. T., Potter, R. L., and Elvehjem, C. A. The phenylalanine and tryptophan content of meats. *Arch. Biochem.*, **15**, 459 (1947).
3. Greenhut, I. T., Schweigert, B. S., and Elvehjem, C. A. The amino acid composition of *Streptococcus faecalis* and the use of this organism for the determination of threonine in natural products. *J. Biol. Chem.*, **162**, 69 (1946).
4. Greenhut, I. T., Sirny, R. J., and Elvehjem, C. A. The lysine, methionine and threonine content of meats. *J. Nutrition*, **35**, 689 (1948).
5. Greenwood, D. A., Kraybill, H. R., and Schweigert, B. S. Amino acid composition of fresh and cooked beef cuts. *J. Biol. Chem.*, **193**, 23 (1951).
6. Hier, S. W., Graham, C. E., Friedes, R., and Klein, D. The microbiological determination of amino acids in animal proteins. *J. Biol. Chem.*, **161**, 705 (1945).
7. Lyman, C. M., and Kuiken, K. A. The amino acid composition of meat and some other foods. *Bulletin 708*, Texas Agric. Exp. Station (1949).
8. Scheid, H. E., Bennett, B. A., and Schweigert, B. S. Thiamine, riboflavin and niacin content of organ meats. *Food Research*, **18**, 109 (1953).
9. Schweigert, B. S., Bennett, B. A., and Guthneck, B. T. Further studies on the amino acid composition of pork and lamb cuts. *J. Biol. Chem.* **190**, 697 (1951).
10. Schweigert, B. S., Bennett, B. A., McBride, B. H., and Guthneck, B. T. Amino acid content of processed meats. *J. Am. Diet. Assn.*, **28**, 23 (1952).
11. Schweigert, B. S., Guthneck, B. T., Kraybill, H. R., and Greenwood, D. A. The amino acid composition of pork and lamb cuts. *J. Biol. Chem.*, **180**, 1077 (1949).
12. Schweigert, B. S., Tatman, I. E., and Elvehjem, C. A. The leucine, valine and isoleucine content of meats. *Arch. Biochem.*, **6**, 177 (1945).

NUTRITIVE VALUE OF MILK PROTEINS.

II. STABILITY DURING STERILIZATION OF EVAPORATED MILK AS DETERMINED BY THE RAT GROWTH METHOD

A. Z. HODSON

Research Laboratories, Pet Milk Company, Greenville, Illinois

(Manuscript received September 19, 1953)

In an earlier paper *(9)* it was stated that sterilization of evaporated milk did not significantly lower the protein nutritive value of the product as measured by the rat nitrogen balance method *(15)*, the dog nitrogen balance method *(14)*, or the rat repletion method *(9)*. It was also stated that Cook, Morgan, Weast, and Parker *(5)* presented data obtained by the rat growth method which suggest the nutritive value of evaporated milk protein is slightly reduced during commercial processing. The data to be presented here, obtained by rat growth method, largely confirm the observations of Cook *et al. (5)*. By supplementation procedures an investigation has also been made as to which amino acids undergo loss or are made unavailable to some extent by the sterilization of evaporated milk.

No attempt will be made to discuss the various methods of biological protein evaluation. Excellent reviews on this subject have been presented by Albanese, Chow, Frost, Mitchell, Porter, and Silber *(1)* and by the Rutgers University Bureau of Biological Research *(13)*. Much has been written concerning the heat damage of proteins. Although it is possible to draw some general conclusions from the data presented, it is obvious that the damage is altered by nature of the product containing the protein, the environmental conditions under which the heating takes place, as well as by the time and temperature of the heating. Minor alterations in the content or availability of the amino acids present in the protein may or may not become apparent according to whether the protein is used for growth, maintenance, repletion, reproduction, or lactation. The value of the protein may also vary depending on whether it is the only source of protein in the diet or is supplemented with other proteins of similar or different amino acid composition.

EXPERIMENTAL

The diet used for most of the experiments is as follows:

Evaporated milk	285.0 g.
Dextrose (Cerelose)	231.0 g.
Trace mineral mixture	1.5 ml.
Jones-Foster mineral mixture (10)	1.5 g.
Vitamin mixture	3.0 ml.
Choline chloride solution (10%)	3.0 ml.

The vitamin mixture used in this diet contains 0.10 g. thiamine, 0.50 g. calcium pantothenate and 1.0 g. niacin amide per l. It supplies about 100 mcg. thiamine, 0.5 mg. calcium pantothenate and 1 mg. niacin amide per 100 g. dry matter when added as indicated above. The vitamins supplied by either non-sterilized or sterilized evaporated milk plus this supplement are adequate to meet the vitamin requirements of the growing rat as stated by Brown and Sturtevant *(4)*. The trace mineral mixture contains: $FeSO_4 \cdot 7H_2O$ — 5 g., $CuSO_4 \cdot 5H_2O$ — 0.63 g., $MnSO_4 \cdot 1H_2O$ — 0.62 g., $CoCl_2 \cdot 6H_2O$

— 0.06 g., KI — 0.04 g., concentrated HCl — 1.0 ml., and water — 200 ml. The diet contains 4.28% protein on a wet basis or 9.00% on a dry basis. Slight variations in the protein and moisture content of the evaporated milk and of the moisture content of the Cerelose cause slight variations from the above figures, but these variations were equalized between groups as will be discussed below.

The male weanling albino rats used in the experiments were purchased from Sprague-Dawley, Madison, Wis., or from Rockland Farms, New City, N. Y. Most of the experiments were 6 weeks in length, but Experiments 1 and 1R were only 4 weeks in length. Except where noted groups of 10 animals were started in each experiment.

Each rat's feed was weighed in daily portions. Any feed remaining after 24 hours was reweighed in order to give accurate consumption records. Since the diets or in the case of Experiment 22 the controlled portion of the diet were all liquid and were fed in automatic test tube feeders there was no scattering of food. Drippage and spillage were kept to a minimum. Although liquid rations were used because of the nature of evaporated milk and the wish to avoid drying and its additional chance of protein alteration, it has been the experience in our laboratory, that the rats tend to waste much less of liquid than of dry rations. In *ad libitum* experiments, every effort was made to provide an excess of the ration each 24-hour period. In controlled feeding experiments only the feed that would be consumed by every rat was fed. In a few cases when a small portion of food remained in the feeding tube, it was weighed and an equivalent amount of fresh diet, together with the usual daily portion, was offered at a later date. In these experiments, the amount of diet offered was controlled by the food consumption of the rat with the poorest appetite. In some cases it was necessary to increase the ration above the poor consumption of a few abnormal rats and to discard the data from these rats. In very hot weather the feeding period was limited to 18 or 15 hours to prevent the milk souring in the feeding tubes.

It was desired that the investigation reported here be made with commercial production of evaporated milk. To do this it was necessary to make the comparison between non-sterilized and sterilized evaporated milk, rather than between fresh milk and sterilized evaporated milk. By making the comparison between the former products it is possible conveniently to avoid all storage problems if the animal experiments can be carried out close to an evaporated milk plant in daily operation. Since the fresh milk at an evaporated milk plant is continuously forewarmed and condensed as received, it is difficult to obtain a representative sample. The concentrated product, however, is mixed and held in large quantities for standardization purposes. After canning it is a simple matter to remove cans from the processing line before and after they pass through a continuous sterilizer. Sterilization, therefore, becomes the only processing factor involved. Sterilization is the most drastic heat treatment evaporated milk receives, but some preliminary heat is applied before the milk goes into the evaporating pan. The evaporation, although carried out at relatively low temperatures under reduced pressure, also involves heat. In the present report we are concerned only with protein changes which occur during sterilizing at 115°-118°C.(239°-244.4°F.) for 14¼ to 14½ minutes.

In our earliest trials non-sterilized and sterilized evaporated milk were compared without supplementation. When the observations of Cook *et al.* *(5)* had been confirmed, diets with a variety of amino acid supplements were tried in order to discover the nature of the alteration which occurs during the sterilization of evaporated milk. Cysteine (free base) was tried first, since the primary deficiency of milk proteins for rat growth appears to be the sulfur-containing amino acids *(8)*. Cysteine was chosen over methionine because it was fallaciously believed the methionine would be difficult to dissolve in the liquid diet. The first cysteine addition was at a level of 1% of the protein. This was later increased to 1½%. After it was shown that cysteine supplementation increased rat growth and the efficiency of protein utilization without entirely eliminating the difference between non-sterilized and sterilized evaporated milk, methionine supplementation was also tested at the higher level. In combination with cysteine a series of other amino acids were tested. These included lysine, histidine, and arginine—all at a level of 1% of the protein—first singly, then in combination. Finally methionine supplementation was tried, first in combination with other amino acids, then alone. Two types of controlled feeding were also tested with methionine

supplementation. In one the rats were allowed free access to a "non-protein" diet and given a limited amount of evaporated milk plus methionine as a protein source. In the other the low protein diet mentioned above plus methionine was fed in limited daily quantities, which all the rats consumed that were considered in the final data. A few rats had abnormally poor appetites; data from these animals were discarded in the latter experiment. The "non-protein" diet used in the former controlled feeding experiment is the one outlined by Frost and Sandy *(7)* with minor modifications. Dextrose was substituted for sucrose, Jones and Foster *(11)*, mineral mixture was substituted for U.S.P. No. 1, and Ruffex for Primex. Ruffex is an aid in the mixing and distribution of the liquids in the diet as well as a source of roughage.

In all cases in which supplements were given comparison was made by supplementing both the non-sterilized and sterilized evaporated milk. This was done to prevent misinterpretation of the data. If the non-sterilized milk was deficient in a particular amino acid, addition of this amino acid to only the sterilized milk might produce additional growth without specifically correcting the damage produced by sterilization. When both types of milk are supplemented, it is essential that sufficient supplement be fed to correct any original deficiency of the milk protein and any loss during sterilization. In exploratory work, estimation of the proper and economic level of supplementation involves some guesswork. After completion of the experiment, however, the data give evidence of whether or not a satisfactory level was chosen.

Over 500 rats were used in the experiments reported here. A number of allied and supporting experiments were also run involving a total of about 1,300 rats. Since about 9,100 animal weighings and 100,000 feed weighings were made, it is obvious that they cannot be reported in detail.

RESULTS

In Table 1 the results of most of the experiments are reported. For simplification, the data are presented in terms of protein efficiency expressed as grams gain per gram protein fed. The protein efficiency data are more uniform than the growth data and since to present both would result in partial duplication only the former are given. The difference in food consumption between the groups of rats fed the non-sterilized and sterilized milk was never large. The arithmetic mean of the per cent difference in food consumption for the experiments recorded in Table 1 was 2.72%. The standard deviation of this mean was ± 1.23. The mean of only the unsupplemented groups was 3% ± 2.31. In the former case the consumption of non-sterilized milk was larger. In the latter case the consumption of sterilized milk was greater.

Experiments 1, 1R, 11, and 16 show that with unsupplemented milk the protein efficiency of the non-sterilized milk is slightly superior to the sterilized milk. Analysis of variance was used to test the significance of the data. Statistical significance is judged at the 5% level. With 10 rats per group, the difference is too small to be significant at the 1% level in most of the comparisons. These data largely confirm those of Cook *et al. (5)*. The trials with cysteine were immediately productive in showing that the milk proteins were deficient in the sulfur amino acids for rat growth. These trials also showed that cysteine supplementation did not eliminate the difference between non-sterilized and sterilized milk although the difference is slightly less with cysteine supplementation.

Further supplementation with lysine, histidine and arginine alone or in combination failed to eliminate the difference in protein efficiency for rat growth between the non-sterilized and sterilized evaporated milk. In general, these amino acids appeared to have little effect. An exception is histidine. Histidine supplementation seemed to depress the protein efficiency of non-sterilized milk and to improve the protein efficiency of the sterilized milk, so that the protein efficiencies of both groups were very similar. However, the protein efficiency of the sterilized milk usually fell below that of the non-sterilized milk without histidine supplementation.

Methionine supplementation was first tried in combination with arginine, histidine and lysine. Although much less soluble than cysteine (free base), methionine was readily dissolved or dispersed in the feed mixture. The uniformity of the rat growth response indicated uniform distribution. Moreover, better protein efficiency was secured than with cysteine in the same mixture. When methionine was tried as a single supplement and in combination with arginine, histidine, lysine and tryptophane (0.5% of protein level) the protein efficiency of methionine alone was nearly as great as with

TABLE 1

A comparison of the protein efficiency for rat growth of non-sterilized and sterilized evaporated milk with and without amino acid supplements

Experiment number	Group number	Supplement	Grams gain per gram protein fed		Difference from paired control		Difference from group one (non-sterilized)	
			Non-sterilized evaporated milk	Sterilized evaporated milk	In per cent	Statistically significant	In per cent	Statistically significant
1	1 & 2	None	2.68	2.39	10.8	Yes	10.8	Yes
1R	1 & 2	None	2.99	2.58	13.7	Yes	13.7	Yes
11	1 & 3	None	2.64	2.40	9.2	Yes	9.2	Yes
	2 & 4	Cysteine	3.02	2.91	3.6	No	10.2	Yes
12	1 & 3	Cysteine	3.25	2.88	8.3	Yes	8.3	Yes
	2 & 4	Cysteine & Lysine	3.22	3.09	4.0	Yes	4.9	Yes
13	1 & 3	Cysteine	3.37	3.08	8.6	Yes	8.6	Yes
	2 & 4	Cystiene & Histidine	3.18	3.20	0.6*	No	5.0	No
14	1 & 4	Cysteine	3.25	2.77	17.3	Yes	17.3	Yes
	2 & 5	Cysteine & Arginine	3.20	2.96	7.5	Yes	8.9	Yes
	3 & 6	Cysteine, Arginine, and Histidine	3.04	3.02	0.6	No	7.1	No
15	1 & 4	Cysteine	3.32	3.05	8.1	Yes	8.1	Yes
	2 & 5	Cysteine, Arginine, Histidine & Lysine	3.13	3.06	2.2	No	7.8	Yes
	3 & 6	Methionine, Arginine, Histidine & Lysine	3.46	3.15	8.9	No	5.1	No
16	1 & 4	None	2.67	2.20	17.6	Yes	17.6	Yes
	2 & 5	Methionine	3.29	3.20	2.7	No	19.8*	Yes
	3 & 6	Methionine, Arginine, Histidine, Lysine & Tryptophane	3.30	3.23	1.1	No	20.9*	Yes
17	1 & 2	Methionine	3.16	3.05	3.5	No	3.5	No

* Sterilized milk group exceeds control.

the combination. The difference in protein efficiency between non-sterilized and sterilized evaporated milk is largely eliminated by the methionine supplementation. The small difference that remains does not have statistical significance as measured by analysis of variance.

In Table 2 the data concerned only with supplementation with cysteine and methionine are tabulated. The comparisons were made on the basis of per cent difference in protein efficiency for the groups fed non-sterilized and sterilized evaporated milk. This basis of comparison avoids certain minor variations between experiments such as source of rats, initial weight of the rats, animal room temperature, etc. The data demonstrates that while cysteine supplementation reduces the difference in protein efficiency between non-sterilized and sterilized evaporated milk, methionine supplementation reduces it even more effectively. This suggests that the more heat stable methionine as well as cystine or cysteine may undergo loss or otherwise be made nutritionally less available by the heat treatment.

Although methionine supplementation reduces the difference in protein efficiency between non-sterilized and sterilized evaporated milk, it does not entirely eliminate it. Other investigations, which were running concurrently, suggested that the difference might be caused by decreased palatability of the sterilized milk, even though the differences in food consumption were known to be small. Differences in palatability directly

TABLE 2

Effect of cysteine and methionine supplementation on the difference in protein efficiency for rat growth between sterilized and non-sterilized evaporated milk

Experiment number	Grams gain per gram protein fed		Difference in per cent
	Non-sterilized evaporated milk	Sterilized evaporated milk	
No Supplement			
1	2.68	2.39	10.8
1R	2.99	2.58	13.7
11	2.64	2.40	9.2
16	2.67	2.20	17.6
Average			12.7
Cysteine Supplement			
11	3.02	2.91	3.6
12	3.25	2.88	8.3
13	3.37	3.08	8.6
14	3.25	2.77	17.3
15	3.32	3.05	8.1
Average			9.2
Methionine Supplement, *ad libitum* feeding			
16	3.29	3.20	2.7
17	3.16	3.05	3.5
Average			3.1
Methionine Supplement, restricted feeding			
22	2.96	2.84	4.0
23	3.09	3.02	2.3
Average			3.1
Average (all methionine supplement)			3.10

affect growth rate but this may be reflected in the protein efficiency. Experiment 22 was planned to control protein intake but allow *ad libitum* intake of a non-protein diet. In this experiment the 2 groups consisted of 28 and 30 rats; both groups received methionine supplements. The results are partially recorded in Table 2. The difference in protein efficiency between non-sterilized and sterilized milk is only slightly higher than is shown for *ad libitum* feeding in Experiments 16 and 17. However, in the case of Experiment 22 the difference is statistically significant at the 5% but not at the 1% level as measured by analysis of variance. The fact that these data were significant may be partially accounted for by unusually uniform response and the large groups of animals used.

Experiment 23 differs from the *ad libitum* trials only in the fact that the amount of ration offered each rat was limited and was uniform among the rats. The results are reported in Table 2. The difference in protein efficiency between the non-sterilized and sterilized evaporated milk is not statistically significant as measured by analysis of variance. Results from the 3 types of rat growth experiments agree in showing that methionine supplementation reduces the difference in protein efficiency for rat growth between the non-sterilized and sterilized milk to a low figure (2.3 to 4.0%) which in most cases has no statistical significance.

DISCUSSION

Like the data of Cook *et al. (5)*, the data presented here, obtained by the rat growth method, show a loss of protein nutritive value produced by

the sterilization of evaporated milk. These data differ from data obtained by nitrogen balance and rat repletion methods. The fact that the sterilization change can be largely corrected by methionine supplementation gives rise to an hypothesis as to why these biological methods do not agree. The rat and dog are covered with hair, a substance with a high content of sulfur amino acids. Their relative requirement for cystine and methionine are therefore higher than for man, who does not have as complete or heavy a hair covering. Rat growth is relatively rapid; during the early growth period the increase in surface area and in the length of the hair is very marked. Therefore, it could be expected that when evaporated milk protein is fed at a low level the deficiency of cystine, cysteine, and methionine is the primary factor limiting growth—as has been observed by other investigators. Hair growth continues throughout life and the adult rat's requirement for cystine, cysteine, and methionine is relatively high compared to that of man. However, when one considers the rat repletion data published earlier, it should be remembered that the protein lost during the depletion period and which is later replaced comes from tissue protein since the hair protein is removed from the metabolic pool as the hair shaft grows. What fluctuations in hair growth and relative methionine requirements may occur during the depletion-repletion cycle are not known.

The general adequacy of casein, enzymatic casein hydrolysates, lactalbumin, and evaporated milk proteins in the sulfur amino acids for the human adult and infant are discussed in papers by Albanese *(1)*, by Cox, Mueller, Elman, Albanese, Kemmerer, Barton, and Holt *(6)*, by Albanese, Holt, Davis, Snyderman, Lein, and Smetak *(2)*, by Albanese, Holt, Irby, Synderman, and Lein *(3)*, and by Mueller and Cox *(12)*. The critical rat growth method indicates, when the results from two laboratories are considered, only about 10% loss in protein efficiency. In view of the data presented and cited here, however, this loss is not applicable to human nutrition.

Evaporated milk is used predominantly as a human food; since the rat growth data on evaporated milk protein are apparently not applicable to human nutrition, there might seem to be little need for investigations such as are reported here. However, at the beginning of the work the nature of the change brought about by sterilization was unknown, and the fact that part of the change occurred in the cystine and methionine could not be predicted. The fact that all of the major laboratory experimental methods of protein evaluation, i.e. amino acid analyses *(10)*, rat nitrogen balance experiments *(15)*, dog nitrogen balance experiments *(14)*, rat repletion (9), and rat growth, show little or no protein damage during the sterilization of evaporated milk is convincing evidence that evaporated milk protein may be freely substituted for fresh milk protein under all types of conditions.

SUMMARY

It appears that the demonstrated amino acid deficiency of evaporated milk proteins for rat growth involves mainly the sulfur-containing amino acids (cystine, cysteine, and methionine). The data suggest that this deficiency may be enlarged slightly by the sterilization of evaporated milk

and possibly, also, that there may be destruction or loss of availability of the methionine as well as the more labile cystine and cysteine. These data should not be projected to other species or to the rat under other conditions than growth. Inasmuch as the requirements of the rat and the human for the sulfur-containing amino acids differ widely, the data do not appear applicable in the nutrition of either the infant or adult human.

Acknowledgments

The author thanks the officials of the Pet Milk Company for releasing this report for publication. He also thanks E. A. Louder, Technical Director, and H. E. O. Heineman, Director of Research Development, for reviewing the manuscript. The laboratory assistance of Roberta Biggins is gratefully acknowledged.

LITERATURE CITED

1. Albanese, Anthony A., Editor; Albanese, Anthony A., Chow, Bacon F., Frost, Douglas V., Mitchell, H. H., Porter, Curt C., and Silber, Robert H. Contributors. *Protein and Amino Acid Requirements of Mammals.* 1950, Academic Press, Inc., New York, N. Y.
2. Albanese, Anthony A., Holt, L. E., Davis, Virginia I., Snyderman, Selma E., Lein, Marilyn, and Smetak, Emilie M. The sulfur amino acid requirement of the infant. *J. Nutrition*, **37**, 511 (1949).
3. Albanese, A. A., Holt, L. E., Irby, V., Synderman, S. E., and Lein, M. Studies on the protein metabolism of the infant. I. The comparative biological value of some milk protein preparations. *Bull. Johns Hopkins Hospital*, **80**, 149 (1947).
4. Brown, R. A., and Sturtevant, M. The vitamin requirement of the growing rat. *Vitamins and Hormones*, **7**, 171 (1949).
5. Cook, Bessie B., Morgan, Agnes Fay, Weast, Elsie O., and Parker, Janice. The effect of heat treatment on the nutritive value of milk protein. I. Evaporated and powdered milks. *J. Nutrition*, **44**, 51 (1951).
6. Cox, Warren J., Jr., Mueller, Arthur J., Elman, Robert, Albanese, Anthony A., Kemmerer, K. S., Barton, R. W., and Holt, L. Emmett, Jr. Nitrogen retention studies on rats, dogs and man: The effect of adding methionine to an enzymatic casein hydrolysate. *J. Nutrition*, **33**, 437 (1947).
7. Frost, Douglas V., and Sandy, Harry R. Assay of dry protein by rat repletion method, nutritive value and amino acid composition of six reference proteins. *J. Nutrition*, **39**, 427 (1949).
8. Greaves, Ethelyn O., Morgan, Agnes Fay, and Loveen, Minnette K. The effect of amino acid supplements and of variations in temperature and duration of heating upon the biological value of heated casein. *J. Nutrition*, **16**, 115 (1938).
9. Hodson, A. Z. Nutritive value of milk proteins. I. Stability during sterilization and storage of evaporated milk as determined by the rat repletion method. *Food Research*, **17**, 168 (1952).
10. Hodson, A. Z., and Krueger, G. M. Essential amino acid content of casein and fresh and processed cow's milk as determined microbiologically on hydrolysates. *Arch. Biochem.*, **10**, 55 (1946).
11. Jones, J. H., and Foster, C. A salt mixture for basal diets either high or low in phosphorus. *J. Nutrition*, **24**, 245 (1942).
12. Mueller, Arthur J., and Cox, Warren M., Jr. Comparative nutritive value of casein and lactalbumin for man. *J. Nutrition*, **34**, 285 (1947).
13. Rutgers University, Bureau of Biological Research. Cooperative determinations of the amino acid content and the nutritive value of six selected protein food sources. New Brunswick, N. J. (1950).
14. Schroeder, Lawrence J., Iacobellis, Michael, and Smith, Arthur H. Heat processing and nutritive value of milk and milk products. *Federation Proc.*, **10**, 393 (1951).
15. Whitnah, Carrel H. Nutritive value of milk protein. *Food Research*, **8**, 89 (1943).

COMPOSITION OF CERTAIN SPECIES OF FRESH-WATER FISH.

I. INTRODUCTION: THE DETERMINATION OF THE VARIATION OF COMPOSITION OF FISH

M. E. STANSBY

Chief, Pacific Coast and Alaska Technological Research, Fish and Wildlife Service, Seattle, Washington

(Manuscript received June 30, 1953)

The chemical composition of fish varies widely from species to species. A thorough knowledge of such composition is necessary if we are to make the best utilization of this natural resource and to be able to process it into food and by-products without waste or loss of important constituents.

Fish are utilized chiefly as a food, primarily for human consumption but, to a lesser extent, in the nutrition of animals as well. A knowledge of the chemical composition with respect to nutritive value is important in order that fish can compete with other sources of animal protein foods such as meat and poultry products. Such information is important to the fishing industry in advertising its products. Chemical composition is also a subject of importance to the housewife since different fish cookery methods are applicable to species of widely differing composition.

Whereas fish make up only a small proportion of the animal protein in most human diets, in some animal diets, fish may be the principal constituent. In such instances, a rather complete knowledge of the chemical composition is vital, if the nutritional requirements of the animal are to be provided.

In processing fish for human consumption, a knowledge of the chemical composition is important in determining the most suitable processing methods to be applied. Thus, in storage of frozen fish a great deal of the difficulty that may be encountered will depend upon the nature and extent of the oils present in the flesh especially with respect to their susceptibility to oxidation. In canning and in drying of fish, the presence of minute amounts of carbohydrate may result in browning (Maillard reaction between sugars and protein).

When fish waste is processed into meal and oil, the selection of the most suitable processing technics will depend to a large extent upon the oil content of the raw material. Need for special precautions in the processing will also depend upon the content of various unstable constituents such as certain of the vitamins, and unless some knowledge of chemical composition is available, an inefficient recovery of such substances may be obtained.

Fish contain many important substances quite aside from the main components of protein, fat, moisture, and ash. Some of these, to mention just a few that have been utilized in the past, include vitamins A and D, cholesterol, lecithin, insulin, fish sounds, and guanine (pearl essence). Undoubtedly, a better knowledge of the composition of fish might reveal a large number of important constituents that could be recovered from fish waste which are not now utilized, and whose presence may not even be suspected.

In the series of papers of which this is the first, attention will be focussed primarily on the gross chemical composition of fish, i.e., on nitrogen (or protein calculated from nitrogen content), moisture, oil, and ash content; some tests as to the nature of the oil with respect to its susceptibility to oxidation will be included.

VARIATION IN COMPOSITION

The chemical composition of fish varies not only from species to species, but often to an even greater extent from one individual fish to another of the same species. For example, the oil content of the common mackerel *(Scomber scombrus)* may vary by 30-fold, and the vitamin A content of the liver of the common gray shark *(Squalus suckleyi)* by more than 1,000-fold. This variation among individuals of the same species may be due to season of the year at which fish are caught, geographical area in which fish are located, age of the fish or other unidentifiable causes. With such wide range of composition of fish occurring, an average value of composition of any given species has little meaning. Of more importance is the establishment of the range of composition values and, if possible, correlation of ranges of values with important factors such as season of year when the fish are caught or geographical areas in which the fish are located.

COMPOSITION DATA IN LITERATURE

A great mass of data on chemical composition of fish has accumulated over a period of years. Thus, in the Netherlands, in a recent literature search, Van der Rijst *(11)* has compiled a bibliography of 436 references in this field; another recent survey *(3)* lists 361 references. Unfortunately, the vast majority of the workers in this field have more or less completely neglected the variation in chemical composition of fish and, in most instances, have taken such an inadequate sample that their results are quite meaningless with respect to determination of either an average value or range of values for any species. Such reported values are merely an analysis of a particular batch of fish, and, especially where no information is reported (as is often the case) as to the source of the samples, the composition data are worthless to other workers.

Undoubtedly the most comprehensive analyses of the chemical composition of fish ever carried out are those of Atwater *(2)* who, from 1880 to 1890 at Wesleyan University in Connecticut with his students, carried out thousands of analyses on the gross chemical composition of a great number of species of American food fish and shellfish. Although these analyses were carried out long ago, the chemical procedures employed are, for the most part, beyond reproach, and the laboratory technics were probably carried out more carefully than much of the later work. As an example of the methods used, moisture was determined by drying in an atmosphere of hydrogen. In spite of these careful and still adequate laboratory procedures, Atwater *(2)* did not recognize the need for adequate sampling, and many of his analyses were carried out on a single individual fish. In other instances where a composite sample of a number of fish was used, the sample was taken at a time of year when the composition was not typical of the bulk of fish landed commercially. Fortunately, Atwater *(2)* reported complete details of the history of all samples he analyzed, so that anyone can readily determine the practical significance of any of the values given. Unfortunately, however, subsequent compilers of data on nutritive values of foods have indiscriminately quoted the data of Atwater *(2)* as typical for the various species and many entirely erroneous values have crept into the literature.

A good example of how this same disregard of the great variation in composition from fish to fish is still resulting in entirely erroneous results appearing in the literature is the more recent data of Sautier *(7)* on riboflavin cited in the 1950 edition of Tressler and Lemon *(9)*. Sautier *(7)* compiled a table on the riboflavin content of fish and shellfish listing average values and range of values for the different species. For 13 of the species reported, the range of values was determined by analyzing just 2 samples, and this is clearly indicated in Sautier's report. For example, Sautier *(7)* analyzed just 2 individual red rockfish for riboflavin, and found values of 72 and 132 mcg. per 100 g. of flesh. This appeared in his table of values as: range of riboflavin

in red rockfish 72-132 and average value 111 meg. per 100 g. These data were then quoted by Tressler and Lemon *(9)* as though they were the true range and average values for this species without mention of the fact that they were based on analysis of only 2 specimens (a completely nonrepresentative sample when a spread of nearly 100% occurred in the riboflavin content). Thus, these probably totally erroneous values have become a part of the permanent literature.

SCOPE OF PROJECT

The Fish and Wildlife Service, realizing the inadequacies of existing data on composition of fish, has set up a project to collect more representative data on this subject. In considering the different types of fish for which such information was needed, it was apparent that less comprehensive and reliable data were available for fresh-water species than for other types. Although considerable work has been carried out at Michigan State College *(4, 5, 6, 10)* on the content of certain vitamins and amino acids in lake fish, data on gross composition of such fish are still very meager. In recent years the laboratories of the Fish and Wildlife Service and of other agencies have collected, incidental to other projects, considerable data on the chemical composition of marine fish. Owing to the location of these laboratories along the coasts, analyses have been restricted to marine fishes. Furthermore, those few analyses of Atwater *(2)* that were made on adequate samples were, in most instances, made on marine fishes; most of his analyses of fresh-water species were made on a single specimen or otherwise inadequate sample. Hence, there has been an acute need for data on the gross chemical composition of fresh-water fish.

Of the constituents, moisture, protein, oil, and ash, the oil content of fish varies to a far greater extent than any other constituent. In some species the variation from one individual fish to another may amount to as much as 30-fold, and variation in average values from one species to another may amount to as much as 100-fold. Other of the above listed constituents never vary by more than about 1½ times. These points were kept in mind in planning the analyses to be carried out on fresh-water fish.

Factors to be considered in the composition determinations include species of fish, season at which fish are taken, and geographic area where fish are caught. The analyses are being carried out on the edible flesh and, in some instances, upon the trimmings and other waste. Determinations of total nitrogen, oil, moisture, and ash are being made, and a measure of the susceptibility toward oxidation of the oil in the flesh is being carried out by storing frozen samples at 0°F. and carrying out organoleptic tests at suitable storage intervals.

It is planned to run analyses for each of the constituents listed above on 16 individual fish of each of the most important species taken commercially at one season and in one locality. Additional individual analyses will be run for oil content of samples taken at different seasons and at different localities. In order to save time other constituents will be determined on composite samples taken at different seasons and in different areas.

The first series of samples taken were of 5 species of Lake Erie fish landed in the fall of 1951 at Sandusky, Ohio. Complete results of these analyses on 16 individual fish of each species will be reported in part II of this series of reports. Preliminary findings have shown that, of the 5 species sampled, whitefish and sheepshead have a considerable and variable amount of oil in the flesh. These species will be analyzed individually for oil content of samples taken in other of the Great Lakes (and, in the case of sheepshead, in rivers). The other constituents of these fish and all constituents of the other species will be analyzed on single composited samples taken at other times and in other areas.

EXPERIMENTAL PROCEDURES

Analytical procedures being used in this work are those described by the A.O.A.C. *(1)*. For oil analysis, the A.O.A.C. procedure *(1)* involving acid hydrolysis and solvent extraction employing the Mojonnier equipment is being used.

Organoleptic examination for susceptibility of the oil toward rancidification is being carried out by a laboratory tasting panel employing technics described by Stansby *(8)*.

In preparing the samples for analysis, the principal analyses are carried out on the skin-and-bone-free fillets. The whole fish is filleted by the usual commercial technic by laboratory workers who are reasonably skilled at this procedure. The fillets are skinned,

taking care to retain as much as possible of the oil deposited directly beneath the skin. The 2 fillets from a single fish are ground through a Hobart food cutter for 6 minutes, the machine being stopped after each 2-minute period, and the contents of the cutting bowl thoroughly mixed. After the last period in the cutter, the fish flesh, which is in an exceedingly fine state of subdivision, is given a final thorough mixing, and is sealed in ½-pound tin cans in a vacuum closing machine, frozen and stored at 0°F. until ready for analysis. For analysis, one can is removed from cold storage, and before being opened is thawed by immersion in cold water. The contents are again very thoroughly mixed and samples weighed for moisture, protein, oil, and ash determinations.

In dealing with extremely small fish such as smelt, where the quantity of flesh is insufficient to follow the above described sampling procedure, a somewhat different technic is employed. Bone-and-skin-free flesh from a single fish which may amount to only a few grams, is frozen and then cut into small pieces with a knife, mixed, and analyzed. In some cases, flesh from a single fish is not sufficient to carry out all analyses. In such instances oil and protein are determined on one fish and ash and moisture on another.

The weight, length, and fillet yield of the fish being analyzed are recorded.

During the course of the analyses and cold storage examination, observations are being made with respect to any technological problems in the handling of any given species. For example, in some of the species for which tests are already under way, it has been observed that oxidation of the oils seems to occur at a rapid rate or a discoloration of the flesh makes the appearance undesirable. Such observations are being carefully noted and may form the basis for future investigations in an attempt to work out improved handling or processing methods.

LITERATURE CITED

1. Association of Official Agricultural Chemists. *Official and Tentative Methods of Analysis.* Association of Official Agricultural Chemists, 7th ed., 1950, Washington, D. C.
2. Atwater, W. O. The chemical composition and nutritive values of food fishes and aquatic invertebrates. *U. S. Fish Commission Report 1888*, 679-868 (1892).
3. Congrés International d'Etude Sur Le Rôle Du Poisson Dans L'Alimentation, 13-108, Paris, October, 1950.
4. Ingalls, Ruth L., Klocke, Jessie Finley, Rafferty, Joan Plummer, Greensmith, R. Ellen, Chang, Miranda Li, Tack, Peter I., and Ohlson, Margaret A. Nutritive value of fish from Michigan waters. Michigan State College Agricultural Experiment Station, Department of Food and Nutrition, Zoology, and the Conservation Institute, *Technical Bulletin 219*, 1-24 (May, 1950).
5. Klocke, Jessie Finley, Porter, Thelma, Tack, P. I., Leffler, Edna, Henry, Norma Scott, and Nitchals, Ruth. Nutritive value of fish from Michigan waters. I. Nicotinic acid of lake herring, carp, common suckers, and burbot. *Food Research*, **11**, 179-86 (1946).
6. Klocke, Jessie Finley, Tack, Peter I., Ohlson, Margaret A., Nitchals, Ruth, Leffler, Edna, and Henry, Norma Scott. Nutritive value of fish from Michigan waters. II. Thiamin of lake herring, carp, common suckers, burbot, and smelt. *Food Research*, **12**, 36-43 (1947).
7. Sautier, Philip M. Riboflavin assays of fishery products. *Commercial Fisheries Review*, **8**, No. 3, 19-21 (March, 1946).
8. Stansby, M. E. Conducting organoleptic tests in the laboratory. *Commercial Fisheries Review*, **13**, No. 11a, 44-6 (November Supplement, 1951).
9. Tressler, Donald K., and Lemon, James M. Marine products of commerce. 2nd ed., 1951, Reinhold Publishing Co., New York.
10. Tack, Peter I., Ingalls, Ruth, Klocke, Jessie Finley, Baeder, Helen, Cedarquist, Dena C., Musser, Elizabeth, and Ohlson, Margaret A. Preparation, utilization, and vitamin concentration of the burbot *(Lota lota maculosa)* (Lesueur). Michigan Agricultural Experiment Station, *Quarterly Bulletin*, **29**, No. 4, 286-93 (1947).
11. Van der Rijst, M. P. J. Literatuuroverzicht over de chemische samenstelling en de voedingswaarde van vis, schaaldieren, en weekdieren. *Voeding*, **11**, No. 6, 5-82 (June, 1950).

BIURET METHOD FOR SOLUBLE WHEY PROTEINS IN NONFAT DRY MILK SOLIDS[a,b]

HAROLD SALWIN
Quartermaster Food and Container Institute for the Armed Forces, Chicago, Illinois

(Manuscript received September 19, 1953)

One of the most important of the factors which influence the baking quality of nonfat dry milk solids is adequate heat treatment of the skim milk before drying. In the manufacture of nonfat dry milk solids for beverage use, however, it is desirable to avoid excessive heat which induces changes in milk proteins associated with cooked flavor, rough tactual quality, and reduced solubility of milk powder. Accordingly, the military specifications for beverage quality nonfat dry milk solids have long required that this ingredient of recombined milk be manufactured under mild conditions of heating. This requirement creates a need for a laboratory test which will serve as an indication of the extent of heat treatment.

The undenatured protein content has been suggested as such an index, and Harland and Ashworth *(9)* developed a very useful turbidimetric method for the estimation of whey proteins. The results of collaborative tests by that method, however, were not always in close agreement. It was thought that a colorimetric method might lend itself toward greater reproducibility, and so the biuret reaction was used for replacing the turbidity measurement by colorimetry. The influence of non-protein constituents of the whey upon the reliability of the method was investigated. The major interference was caused by lactose which exhibited the properties of a polyhydric alcohol by reacting with the copper sulfate and sodium hydroxide of the biuret reagent to form a dark-blue complex. Its reducing action on the copper sulfate was an additional source of error. The method which was developed corrects for the interference by lactose without the necessity for a time-consuming lactose-removal operation. The method is simple but close attention to its details is necessary for best results. This paper is confined to a description of the method, the steps in its development, and its application to nonfat dry milk solids of known preheat treatment.

MATERIALS AND METHODS

Equipment. Constant temperature bath at 40° ± 1°C.

Centrifuge for operation at 2,000 to 2,400 r.p.m. (International Equipment Company[c] size No. 2, equipped with No. 831 and No. 845 angle heads, was used.)

[a] Presented at the Twelfth Annual Meeting of the I.F.T., Grand Rapids, Mich., June 11, 1952.

[b] This paper reports research undertaken by the Quartermaster Food and Container Institute for the Armed Forces and has been assigned No. 378 in the series of papers approved for publication. The views or conclusions contained in this report are those of the author. They are not to be construed as necessarily reflecting the views or indorsement of the Department of Defense.

[c] The mention of this and other commercial products does not imply that they are endorsed or recommended by the Department of Defense over similar products not mentioned.

Spectrophotometer. (Coleman Universal Model 14 with 13-mm. square cuvettes was used.)

Test tubes, 25 × 150 mm., fitted with No. 5 rubber stoppers.

Centrifuge tubes with constricted necks, 100-ml. capacity (Corning No. 8460).

Conical centrifuge tubes, 15-ml. capacity (Corning No. 8060 or 8080), fitted with No. 1 rubber stoppers.

Rubber caps for 15-ml. centrifuge tubes (International Equipment Company No. 580.)

Filter paper, Whatman No. 2, 11-cm. and 24-cm. diameter.

Reagents. Sodium chloride, granular, reagent grade.

Saturated sodium chloride solution, prepared by mechanically stirring for at least 30 minutes a mixture of 370 g. of reagent grade granular sodium chloride and 1,000 ml. of distilled water.

Lactose-sodium chloride solution, prepared fresh daily by dissolving 4.450 g. of reagent grade lactose monohydrate in saturated sodium chloride solution and diluting to 100 ml. with saturated sodium chloride.

Trichloroacetic acid solution (7.5% TCA), prepared fresh daily by dissolving 7.5 g. of reagent grade TCA in distilled water and diluting to 100 ml.

Sodium hydroxide solution (3%), prepared fresh daily by dissolving 7.5 g. of reagent grade sodium hydroxide pellets in 25 ml. of distilled water, cooling, and diluting to 250 ml.

Copper sulfate solution (5%), prepared fresh daily by dissolving 2.50 g. of reagent grade cupric sulfate pentahydrate in 35 ml. of distilled water and diluting to 50 ml.

Procedure. The procedure is presented schematically in Figure 1. The casein-free filtrate, hereafter referred to as "whey," is prepared according to the procedure of Harland and Ashworth *(9)*. Weigh 8.0 g. of sodium chloride into a 25 × 150 mm. test tube and add 2.00 g. of nonfat dry milk solids. Pipette 20 ml. of distilled water into the tube; stopper the tube, shake it vigorously with a vertical motion for 40 seconds,

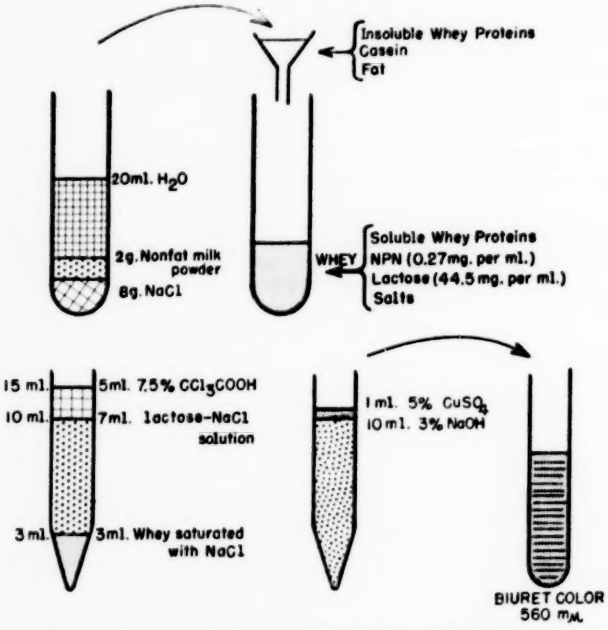


Figure 1. Diagram of the biuret method for whey proteins.

and immerse it, without further agitation, in a 40°C. water bath for 30 minutes. Filter the mixture through an 11-cm. Whatman No. 2 paper into a test tube. The funnel should be supported by the receiving tube and covered with a watch glass. Stopper the receiving tube and swirl to mix contents. The filtrate can be held until the following day, if desired, but it should be held at room temperature.

Using a volumetric pipette, transfer a portion of the whey to a 15-ml. conical centrifuge tube. Three ml. of whey are sufficient in all cases. However, if the sample is known or suspected to have had high-heat treatment, it is preferable to use a 10-ml. aliquot of the whey. If less than 10 ml. are used, dilute the whey to 10 ml. with lactose-sodium chloride solution. Add 5 ml. of 7.5% trichloroacetic acid (TCA) solution and, without delay, stopper the tube and invert it gently 5 times in about 10 to 15 seconds total time. The motion should be gentle and at uniform rate with insufficient agitation to start flocculation. The rubber stopper should fit the tube tightly with no dead space around the rim and leave an enclosed volume of at least 15.5 ml. In some cases it is necessary to shorten a No. 1 rubber stopper by cutting off approximately 5 mm. at the smaller end. Graduations on the tube can be used for measuring the lactose-sodium chloride and TCA solutions, but it is better to measure these with pipettes. Twelve to 14 tubes can be run conveniently in a group. Twenty minutes after adding the TCA to the last tube of a group, remove the stoppers, and centrifuge the tubes for 10 minutes at a speed of 2,000 to 2,200 r.p.m. It is not necessary to balance each pair of tubes at time of use if all the tubes for a day's run are weighed in advance and selected within a tolerance of 0.5 to 0.6 g. of each other. The centrifugation should be done under conditions which prevent evaporation from the tubes. This can be accomplished by closing the bottom opening of the centrifuge guard bowl and wetting the inside of the bowl with water, or by closing each tube with a rubber cap.

Decant the supernatant solution. It is generally perfectly clear. Occasionally, it may have a slight turbidity, but protein flakes should be entirely absent. Invert the tube on a paper towel or filter paper and allow it to drain for 20 minutes. Wipe off the last remaining drop on the rim of the tube with a cleansing tissue and return the tube to an upright position.

Add 10 ml. of 3% sodium hydroxide solution, measured with a volumetric pipette, and stopper the tube with the same stopper used previously. Shake the tube until the precipitate dissolves completely. When the tube is mounted horizontally in a Kahn shaking machine parallel to the direction of travel, approximately 12 to 15 minutes are required. Set the tube in a rack until the foam disappears, generally 30 minutes, or 15 minutes if unstoppered. The tubes may be allowed to stand for a longer period if desired.

Add 1 ml. of 5% copper sulfate solution, measured from a 10-ml. burette, and without delay, stopper the tube with the same stopper used previously and shake it vigorously 10 times with a vertical motion. The shaking procedure should be uniform for all tests, and in the case of a series of tubes each copper sulfate addition and shaking should be completed before proceeding to the next tube. Run a blank determination consisting of 10 ml. of 3% sodium hydroxide and 1 ml. of 5% copper sulfate at the same time. Note the time of addition of copper sulfate to the first tube and make successive additions at 30-second intervals. Remove the stoppers and, 10 minutes after the last mixing, centrifuge the tubes for 10 minutes at a speed of 2,000 r.p.m. in order to settle the precipitate of copper hydroxide. Prevent evaporation from the tubes as in the previous centrifugation.

Decant the solutions into cuvettes and measure the intensity of the colors with reference to the blank at 560 mμ. Make the first reading 40 minutes after the first addition of copper sulfate and continue with succeeding tubes at 30-second intervals. Convert the absorbency readings to milligrams of protein nitrogen by reference to a standard curve, and finally to mg. of whey protein nitrogen per g. of nonfat dry milk solids by multiplying by the factor $\frac{11.75}{\text{ml. of whey tested}}$.

Preparation of standard curve. Select a sample of nonfat dry milk solids of low-heat treatment or high whey protein content. Combine 60 g. of sodium chloride with 15.0 g. of the nonfat dry milk solids and 150 ml. of distilled water in a 500-ml. Erlenmeyer flask, close the flask with a rubber stopper, and shake it vigorously for 40 seconds.

Prepare 3 such flasks and immerse them in a 40°C. water bath for 30 minutes. Filter each mixture through a fluted 24-cm. Whatman No. 2 paper into an Erlenmeyer flask. Cover the funnel with a watch glass and close the mouth of the receiving flask around the funnel stem with paper to minimize evaporation. Combine the 3 filtrates in a single flask, stopper the flask, mix the contents and store at room temperature. The yield of whey is 300 to 350 ml.

For nitrogen determination, pipette aliquots of the whey into 100-ml. centrifuge tubes. The aliquots should be from 25 to 50 ml. in volume, and from 2 to 6 determinations should be made. Dilute each aliquot of whey to 60 ml. with saturated sodium chloride solution and add 30 ml. of 7.5% TCA solution. The sodium chloride and TCA solutions may be measured carefully in 50-ml. graduated cylinders. Stopper the tubes with rubber stoppers and invert them gently 5 times in about 10 to 15 seconds total time. Each tube should be stoppered and inverted immediately after the TCA addition and before proceeding to the next tube. After 20 minutes, centrifuge the tubes at 2,400 r.p.m. Decant the supernatant solution, being careful not to lose any of the precipitate. Add 25 ml. of a wash solution composed of 1 volume of 7.5% TCA plus 2 volumes of saturated sodium chloride solution. Mix the solution and precipitate by swirling the tube gently and by breaking up lumps with a glass rod. Add 60 ml. more of the wash solution, which is used also for washing the glass rod, remove the rod, stopper the tube, and invert it several times. After 20 minutes, centrifuge and decant as before. Subject the precipitate to a second washing of the same kind.

Dissolve the precipitate in 60 ml. of 3% sodium hydroxide solution and transfer quantitatively with washing to an 800-ml. Kjeldahl flask. (Note: If facilities for centrifuging 100-ml. tubes are not available, 10-ml. aliquots of whey can be treated in 15-ml. tubes and the contents of 3 or 4 such tubes combined in a single Kjeldahl flask at this point.) Determine nitrogen by the Kjeldahl procedure and correct for blank determinations on the reagents in accordance with the A.O.A.C. procedures *(1)*. For the digestion use 35 ml. of concentrated sulfuric acid, 10 to 12 g. of anhydrous sodium sulfate, 0.7 g. of mercuric oxide, and glass beads. Digest the mixture for 30 minutes after it is colorless. Express the results as milligrams of nitrogen per ml. of whey.

Prepare 1:10 whey by diluting exactly 5 ml. of whey to 50 ml. with lactose-sodium chloride solution. Prepare 1:1 whey, similarly, by diluting 25 ml. to 50 ml. with lactose-sodium chloride solution. With volumetric pipettes, transfer 0, 1, and 3 ml. of 1:10 whey and 1, 2, 3, 4, 5, 6, 8, and 10 ml. of 1:1 whey to 15-ml. centrifuge tubes. Dilute each aliquot to 10 ml. with lactose-sodium chloride solution, and continue with the procedure described previously. Prepare a standard curve relating absorbency to milligrams of nitrogen.

DEVELOPMENT OF METHOD

Size of sample. Almost identical results were obtained when the method was applied to the whey separated from 1-, 5-, 10-, and 20-g. samples of nonfat dry milk solids. Consequently, it is permissible to make a bulk separation of whey in order to obtain sufficient material for calibration purposes and to use a smaller sample for the test.

Total volume of whey. In computing the mg. of whey protein nitrogen per g. of nonfat dry milk solids in the last step of the analytical procedure, the factor 11.75 represents the volume of whey in ml. obtained from one g. of sample. This value was obtained in two ways. The first was a double-dilution procedure in which lactose was determined in 2 whey solutions of different concentration and different volume by a modification of Cajori's *(2)* iodometric procedure for the determination of aldoses. The second was a gravimetric method in which the total volume of whey was determined by establishing the relationship between volume of whey and weight of water contained in it.

The factor 12.0 used by Harland and Ashworth was apparently determined by the method which they described in an earlier paper *(8)*. In that method, the volume occupied by the precipitated protein was ignored on the basis of Rowland's *(11)* work on the volume occupied by casein and fat separated from whole milk. However, Rowland's factor of 0.995 to correct for the volume of the precipitated casein and fat was arrived at by analysis of filtrates from milk diluted 10-fold. The factor is not applicable to undiluted milk in which case it would be reduced from 0.995 to 0.95. Using the value 1.318 for the density of casein, as reported by Chick and Martin *(3)* and cited later by Cohn and Edsall *(4)*, the volume correction for 1.0 g. of nonfat dry milk solids

is 0.28 ml. The Harland and Ashworth factor reduced by this amount agrees closely with the factor used in this work.

Composition and stability of whey. The major constituents of the whey are listed in Figure 1. The figure for non-protein nitrogen was obtained by subtracting the protein nitrogen from the total nitrogen. These were determined on the whey from a typical low-heat powder by the Kjeldahl method, using TCA for the protein separation as described under "Preparation of standard curve." The value of 0.27 mg. per ml. agrees closely with the amounts reported by others *(8, 9, 11, 12)*. The figure shown for lactose, 44.5 mg. per ml., represents lactose monohydrate. It was determined by the iodometric procedure referred to previously *(2)* and is nearly constant for all samples.

Kjeldahl nitrogen values on a sample of whey determined 8 days apart differed by less than 1%. Variations in biuret determinations 9 days apart were less than 2%. A white precipitate separates from the whey upon prolonged standing. This separation can be minimized by holding the whey at room temperature rather than under refrigeration. A Kjeldahl nitrogen determination on the insoluble material which had separated from a sample of whey after several weeks' standing amounted to 0.04 mg. of nitrogen per g. of the original milk powder.

Volume of whey for analysis. Three ml. of whey from a low-heat sample of nonfat dry milk solids or 10 ml. from a high-heat sample provide an amount of protein which can be measured within the most accurate range of the Coleman spectrophotometer or Evelyn colorimeter. The final result by the biuret method was found to be independent of the volume of whey tested. This was not always the case with the Harland and Ashworth procedure, in which the degree of turbidity, measured in absorbency units, bore a linear relationship with volume of whey tested in some cases, but not in others. This variability with concentration cast some doubt on the reliability of the turbidimetric results.

Lactose-sodium chloride solution. The salt concentration influences the recovery of protein. Dilution of the whey with water rather than with saturated sodium chloride solution reduces the intensity of the final biuret color. The concentration of lactose in the lactose-sodium chloride solution is the same as the concentration of lactose in the whey. Regardless of the amount of whey taken for analysis, after it is diluted to 10 ml. the solution contains 445 mg. of lactose monohydrate. The significance of this is discussed under the heading "Compensating the error due to lactose."

Trichloroacetic acid solution (TCA). The final concentration of TCA is 2.5%. This concentration gave the greatest recovery of protein, and, according to Hiller and Van Slyke *(10)*, is optimum for the separation of protein from non-protein nitrogenous material. As the final concentration was varied from 0 to 20%, a second, but smaller, maximum recovery point occurred at about 13%. Above 13%, the protein separated as an oil from which a solid precipitate could be recovered by shaking with water.

Precipitation of whey protein. In some instances the head space above the 15-ml. mark in the conical centrifuge tubes was so small that it was necessary to shorten the No. 1 rubber stoppers by cutting off approximately 5 mm. from the smaller end. There are a number of advantages in using the full capacity of the tube. Greater accuracy was provided in measuring the largest possible volumes of reagents. Also, dilution of the whey insured greater purity of the protein precipitate. If the tubes contained less than 15 ml. and were unstoppered during centrifugation, drying on the sides above the liquid allowed lactose and other non-protein material to be retained, as well as some protein which may be lost, in the subsequent decantation.

Recovery was best when only gentle mixing with TCA was employed, and the use of tubes with only 0.5 to 1 ml. of air space insured the required gentle mixing. When the tubes were shaken or when the concentration of TCA was high, protein was frequently lost during decantation. Immersing the tube in a boiling water bath for 10 minutes coagulated the precipitate to a non-coherent mass which did not stick to the glass, even after centrifuging.

When the time for coagulation of the protein was varied from 0 to 30 minutes the maximum variation in final color density was 2.5%. The period for optimum recovery was 20 minutes.

Re-solution of whey protein. Approximately 15 minutes on the Kahn shaking machine with 3% sodium hydroxide solution was satisfactory for re-solution of the protein precipitate. There were no apparent advantages in using higher concentrations of

sodium hydroxide or shaking times longer than necessary to effect solution of the protein. Although the concentration of sodium hydroxide affects the intensity of the final color, the concentration selected is not important if it is kept uniform throughout the calibration, tests, and blank determinations.

Development of biuret color. The concentrations and volumes of sodium hydroxide and copper sulfate solutions correspond closely with those used by Fine *(5)*. Gornall, Bardawill, and David *(6)* recommended a biuret reagent containing copper sulfate, sodium potassium tartrate, and sodium hydroxide in a single solution. This "one piece" reagent was tried and discarded because the final solutions were always turbid.

The copper sulfate solution was prepared fresh daily because a precipitate forms in the solution after several days. Somewhat higher results were obtained with a 3-day-old solution.

One ml. of 5% copper sulfate solution appeared to be adequate for color development. Increased amounts of copper sulfate reduced the color intensity, but variations of 10% in the volume added changed the color density less than 1%.

When the period of contact with copper sulfate from the point of addition until the start of centrifugation was varied from 8 to 16 minutes, the variations in color intensity were not more than 2%.

Upon addition of copper sulfate there is an immediate precipitation of cupric hydroxide. Thorough mixing is necessary for complete color development, apparently to effect contact between the remaining copper sulfate and the protein adsorbed by the precipitate. The method of mixing at this point is important inasmuch as it affects the density of the final color. Consistent results between operators were obtained with the procedure described and variations from day to day with one operator did not exceed 2.5%. With more gentle mixing, ranging from 5 inversions of the tube at slow speed to 5 gentle rockings of the tube, results were as much as 14% lower. With more vigorous mixing, from 30 seconds of vertical shaking to 16 minutes on the Kahn shaking machine, results varied by less than 3%, and in no case did this amount to more than 0.12 mg. of nitrogen per g. of powder. Centrifugation of the precipitate should be done under conditions tending to prevent concentration of the solution by evaporation.

Stability of the biuret color. The biuret color fades, but in the period from 30 to 90 minutes after addition of the copper sulfate the color did not fade more than 2.4% for a low-heat sample nor more than 4% for a high-heat sample. The maximum fading encountered during any 10-minute interval between 30 and 90 minutes represented 0.04 mg. of nitrogen per g. of powder.

Effects of non-protein components of the whey. When the whey proteins were precipitated from solution, and the supernatant liquid decanted, portions of the other constituents remained, either adsorbed by the protein or adhering to the walls of the tube. Lactose added to the final color by reacting with the copper sulfate and sodium hydroxide to form a dark-blue complex. The reducing action of the lactose was probably responsible for the subsequent fading of the color discussed in the previous section. Of the non-protein components of the whey, only lactose interfered with the reaction. This is demonstrated by the data in Table 1. The whey proteins were precipitated from a solution made up of 2 volumes of saturated sodium chloride and one volume of 7.5%

TABLE 1

Effect of non-protein components of whey on the biuret reaction

Material reacting with biuret reagent	Biuret color. Absorbency, 560 mμ			
(1) Whey protein (impurities removed by washing precipitate with CCl_3COOH-NaCl solution)	0.400	0.402	0.411	Av. 0.404
(2) Whey protein (not washed)	0.475	0.484	0.486	Av. 0.482
(3) Lactose associated with (2) (determined by analysis)[a]	0.073	0.078	0.080	Av. 0.077
(4) Whey protein, corrected for lactose, (2)-(3)	0.402	0.406	0.406	Av. 0.405

[a] 5.27, 5.78, and 5.91 mg. lactose monohydrate, respectively; average 5.65 mg.

TCA, as in the regular procedure with the exception that the sodium chloride solution used for diluting the whey to 10 ml. contained no lactose. A wash solution of sodium chloride and TCA made up in this proportion was found to be effective in removing impurities from the precipitate without loss of protein. The biuret reaction was applied to the protein precipitated from 3 ml. portions of whey. The impurities were removed from 3 of the precipitates by re-suspending them in 14 ml. of the wash solution and separating again by centrifuging. The remaining 3 precipitates were not purified, and the lactose contained in them was determined by analysis. The amount of color due to lactose was then estimated from a calibration curve for the biuret reaction with lactose. When this figure was subtracted from the color density produced by the crude precipitate, the corrected measurement agreed within 1% of the value obtained with the purified precipitate. This was taken as evidence that lactose is the only non-protein component of the whey which contributes to the biuret color.

In order to demonstrate the magnitude of the errors due to non-protein materials in the whey, the data of Table 1 are presented again in Table 2. The figure 1.69 mg.

TABLE 2

Magnitude of errors introduced by lactose and non-protein nitrogenous materials

Whey component	Amount in 3 ml. whey	Amount in CCl_3COOH precipitate	Biuret color. Absorbency, 560 mμ	Nitrogen equivalent of biuret color
	mg.	*mg.*		*mg.*
Protein	10.8	10.8	0.405	1.69
Lactose	133.5	5.65	0.077	0.34
NPN	0.81	0.05	.....	.....

$$\text{Lactose error} = \frac{0.34}{1.69} \times 100 = 20\%.$$

$$\text{NPN error} = \frac{0.05}{1.69} \times 100 = 3\%.$$

corresponds to absorbency 0.405 on a calibration curve for the biuret reaction with whey protein in which each protein precipitate was purified with a TCA-sodium chloride wash solution. The figure 0.34 mg. corresponds to absorbency 0.077 on the same curve, and represents the nitrogen equivalent, in terms of biuret color, of 5.65 mg. of lactose monohydrate. The figure 10.8 mg. was obtained by multiplying 1.69 mg. by the factor 6.38. These data indicate that the error due to lactose may be as much as 20%. The figure 0.05 mg. for NPN was obtained by difference between Kjeldahl nitrogen determinations on crude and purified protein precipitates. Although this amount is 3% of the total nitrogen, the error in measuring the biuret color would be smaller because the NPN consists of some fractions which give no biuret reaction, and of other fractions which, if they do react, form products which do not have absorption maxima at 560 mμ *(7)*. The protein-washing step described under "Preparation of standard curve" is intended to eliminate the 3% error in Kjeldahl nitrogen determinations.

The possibility of reducing or eliminating the error due to lactose by measuring the color at some other wave length was investigated. Figure 2 demonstrates that in going from 560 to 455 mμ, the color due to lactose is reduced by four-fifths, but the color due to protein is reduced also by two-thirds. A 20% error at 560 mμ would still be an 11% error at the shorter wave length.

Dialysis was tried for removing lactose from the whey. This time-consuming step made the procedure less practicable and led to variable results. The error due to lactose was eliminated by removing the lactose from the precipitated protein with the wash solution described previously and the method gave satisfactory results. However, the extra time required for the purification step made it difficult to complete a large number of determinations in a single day. In the shorter procedure described in this paper, instead of removing lactose, more is added.

Compensating the error due to lactose. The data in Table 3 demonstrate that lactose would produce a variable error if no correction were made for it. Each point on the standard curve would then be made under the condition of a constant lactose-nitrogen

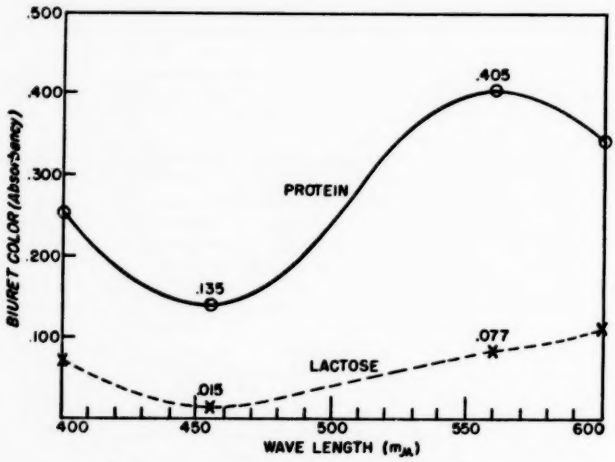


Figure 2. Biuret reaction absorption spectra.

ratio in the whey. If the nonfat dry milk solids chosen for the standard curve contained 5.88 mg. of whey protein nitrogen per g., determined by Kjeldahl, this fixed ratio would be 89. In the examples of sample tests in Table 3, however, the lactose-nitrogen ratio varies from 67 to 445.

Table 4 shows the effect of having 44.5 mg. of lactose monohydrate dissolved in each ml. of saturated sodium chloride solution used for diluting the whey. Each point on the standard curve and each test is then made in the presence of 445 mg. of lactose monohydrate. By this means, the error due to lactose is compensated for and the procedure is appreciably shortened.

Standard curve. The calibration curve for the method was prepared for the Coleman Universal Spectrophotometer, Model 14, with 13 mm. square cuvettes, at 560 mμ. The

TABLE 3

Variability of error due to lactose

Whey protein nitrogen	Standard curve			
	Whey	Saturated NaCl solution	Protein nitrogen	Lactose
mg./g.	*ml.*	*ml.*	*mg.*	*mg.*
5.88	0	10	0	0
	1	9	0.5	44.5
	2	8	1.0	89
	3	7	1.5	133.5
	4	6	2.0	178
	7	3	3.5	311.5
	10	0	5.0	445
	Sample tests			
Low heat				
7.83	3	7	2.0	133.5
3.92	3	7	1.0	133.5
High heat				
1.96	3	7	0.5	133.5
1.18	10	0	1.0	445

TABLE 4

Standardization of error due to lactose

Whey protein nitrogen	Standard curve			
	Whey	Saturated NaCl containing 44.5 mg. lactose per ml.	Protein nitrogen	Lactose
mg./g.	*ml.*	*ml.*	*mg.*	*mg.*
5.88	0	10	0	445
	1	9	0.5	445
	2	8	1.0	445
	3	7	1.5	445
	4	6	2.0	445
	7	3	3.5	445
	10	0	5.0	445
	Sample tests			
Low heat				
7.83	3	7	2.0	445
3.92	3	7	1.0	445
High heat				
1.96	3	7	0.5	445
1.18	10	0	1.0	445

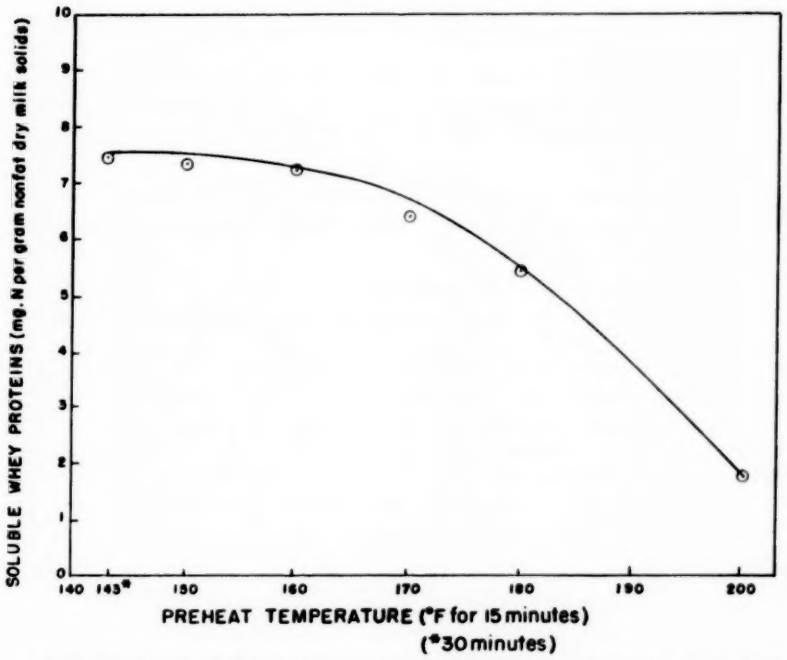


Figure 3. Effect of preheat treatment on soluble whey protein content of nonfat dry milk solids, determined by the biuret method.

regression line, obtained by the method of least squares, is $N = 3.327\ A - 0.090$, in which N is the number of mg. of protein nitrogen in the final solution, and A is the absorbency of the biuret color. The standard error of estimate is 0.018 mg. of nitrogen. For the procedure in which the lactose is removed from the precipitated protein with a wash solution, the equation is $N = 4.125\ A + 0.018$, and the standard error of estimate is 0.016 mg.

APPLICATION OF THE METHOD TO NONFAT DRY MILK SOLIDS

As little as 0.06 mg. of protein nitrogen can be determined. The effect of preheat treatment on the soluble whey protein content of nonfat dry milk solids is illustrated in Figure 3. In applying the method to 24 commercially-prepared milk powders, samples designated as having high-preheat treatment were found to contain less than 2.0 mg. of whey protein nitrogen per g., whereas low-heat samples generally contained from 5.0 to 8.0 mg. of whey protein nitrogen per g. Table 5 summarizes the results

TABLE 5

Analysis of commercial samples of nonfat dry milk solids

Commercial designation of samples	Whey protein nitrogen
	mg. per g.
Low-preheat treatment*	
Average of 12 samples	6.62
Range of 12 samples	5.39 - 7.98
3 samples	1.66, 3.02, 3.80
High-preheat treatment†	
Average of 9 samples	1.13
Range of 9 samples	0.36 - 1.64

*From 165°F. for 30 seconds to 170°F. for 10 to 20 seconds.
†From 175°F. for 30 minutes to 265°F. for 40 seconds.

of these tests which indicate that determinations of soluble whey protein nitrogen provide a fairly reliable differentiation among milk powders of different preheat treatment.

SUMMARY

A biuret method for the determination of soluble whey proteins in nonfat dry milk solids is described. The composition and properties of the whey, the interference by non-protein components, and the analytical details which influence the reliability of the method are discussed. Errors due to the reaction of lactose are standardized and compensated for by a calibration curve. Commercially-prepared nonfat dry milk solids having high-preheat treatment were found to contain less than 2.0 mg. of whey protein nitrogen per g. whereas low-heat samples generally contained from 5.0 to 8.0 mg. of whey protein nitrogen per g.

Acknowledgments

The author acknowledges with appreciation the technical assistance of A. B. Evans, formerly of the Quartermaster Food and Container Institute, and the cooperation of R. J. Remaley, formerly of the Kraft Foods Company, in supplying samples of nonfat dry milk solids of known preheat treatment.

LITERATURE CITED

1. Association of Official Agricultural Chemists. *Official Methods of Analysis.* 7th Ed., 1950, Washington, D. C.
2. Cajori, F. A. The use of iodine in the determination of glucose, fructose, sucrose, and maltose. *J. Biol. Chem.*, **54**, 617 (1922).
3. Chick, H., and Martin, C. J. The density and solution volume of some proteins. *Biochem. J.*, **7**, 92 (1913).
4. Cohn, E. J., and Edsall, J. T. *Proteins, Amino Acids and Peptides.* 1943, Reinhold Publishing Corp., New York, N. Y.
5. Fine, J. The biuret method of estimating albumin and globulin in serum and urine. *Biochem. J.*, **29**, 799 (1935).
6. Gornall, A. G., Bardawill, C. J., and David, M. M. Determination of serum proteins by means of the biuret reaction. *J. Biol. Chem.*, **177**, 751 (1949).
7. Gortner, R. A., Jr., and Gortner, W. A. *Outlines of Biochemistry.* 3rd Ed., 1949, John Wiley and Sons, Inc., New York, N. Y.
8. Harland, H. A., and Ashworth, U. S. The preparation and effect of heat treatment on the whey proteins of milk. *J. Dairy Sci.*, **28**, 879 (1945).
9. Harland, H. A., and Ashworth, U. S. A rapid method for estimation of whey proteins as an indication of baking quality of nonfat dry milk solids. *Food Research*, **12**, 247 (1947).
10. Hiller, A., and Van Slyke, D. D. A study of certain protein precipitants. *J. Biol. Chem.*, **53**, 253 (1922).
11. Rowland, S. J. The precipitation of the proteins in milk. *J. Dairy Research*, **9**, 30 (1938).
12. Shahani, K. M., and Sommer, H. H. The protein and non-protein nitrogen fractions in milk. *J. Dairy Sci.*, **34**, 1010 (1951).

ROPY MILK. I. THE BIOLOGY OF ROPE PRODUCTION[a]

DANIEL L. JONES[b]
Department of Biological Sciences, Duquesne University, Pittsburgh, Pennsylvania

(Manuscript received October 1, 1953)

From a study of the literature, it appears that little new information regarding ropy milk has been added since the excellent review of Hammer and Buchanan *(6)* in 1915. In fact, the subject has received almost no attention for the past decade. This apparent decline in importance parallels a decline in the frequency of ropy milk epidemics which stimulated much of the early work. The present day infrequency of ropy milk outbreaks undoubtedly is due to progress in dairy plant sanitation and practice.

Most investigations have been confined to a determination of the cause and cure of epidemic ropiness in milk. Much remains to be learned regarding the nature of the microorganisms which produce rope, the influence of their chemical and physical environment, and the chemical nature of the ropy substance produced. The present work was begun with the hope of remedying some of these deficiencies in our knowledge.

EXPERIMENTAL METHODS

Standard bacteriological procedures were used in the isolation and identification of bacteria capable of producing rope in milk.

Isolations were made by sampling raw milk at dairy receiving stations, diluting, plating in simple nutrient agar enriched with milk, and incubating 48 hours at room temperature. All mucoid appearing colonies were tested with a straight needle and those evidencing stringiness were cultured in litmus milk for 24 hours at room temperature. Ropiness was tested by withdrawing a wire loop from the surface of the milk culture.

Classification was by standard methods; namely, morphological, cultural, and biochemical studies. The species classified have been described many times from ropy milk epidemics. Therefore, a detailed description here should not be necessary. A brief notation of conspicuous characteristics, with special attention to interesting variational patterns, is given below in order to properly identify cultures used in studies yet to be reported.

RESULTS

From more than 3,500 samples of raw milk, a total of 440 ropy cultures were isolated and identified. These included 3 stable, highly ropy species which are to be discussed below. Weakly or transiently ropy streptococci were encountered, as were species of flavobacterium which produced slime associated with a surface pellicle. These latter two will not be described.

Alcaligenes viscosus (Adametz) was found to be a Gram-negative rod, producing ropiness and alkalinity in milk, as stated in Bergey's Manual *(4)*, and tending to be rather inert with respect to fermentative abilities. Long and Hammer *(7)* noted that Gram-positive cells occur rarely. In the present study, pleomorphism and Gram-positiveness were commonly observed, giving the appearance of a mixed culture under the

[a] In part from a dissertation submitted in partial fulfillment of the requirements for the Master of Science degree at the Pennsylvania State College, State College, Pennsylvania.

[b] Present address: The Hektoen Institute for Medical Research of the Cook County Hospital, Chicago, Illinois.

microscope. A constant fermentative characteristic observed was the inability to attack sucrose. Commonly, cultures were noted to have at isolation or after prolonged storage the ability to ferment glucose slowly, and in some instances lactose as well.

Colonies appeared smooth but not conspicuously mucoid. Such colonies produced ropiness in 24 hours in litmus milk at room temperature, with ropiness progressing from the top downward. Rough variants produced a pellicle in milk and a slimy, alkaline surface but not true ropiness. One culture stored 19 months on nutrient agar developed a citron yellow daughter colony. Subculture of this pigmented colony gave the normal non-pigmented form.

Ropy *Aerobacter aerogenes* differed in no respect from the non-ropy recognized species except in the ability to produce rope. IMVic tests of 37 ropy coliform cultures showed all possible combinations of reactions except those of *Escherichia coli*, which was not found in this study.

To determine whether quantitative biochemical differences exist between ropy and non-ropy strains of *Aerobacter aerogenes*, 5 ropy milk strains and 5 non-ropy fecal strains were cultured in 2% glucose-peptone broth for one week at 37°C., then analyzed for total acetylmethylcarbinol production by the method of Stahle and Werkman *(9)*. Control bacterial counts were made. The results are summarized in Table 1, from

TABLE 1

Acetylmethylcarbinol production by Aerobacter aerogenes

	Average weight of AMC per l.	Average bacterial count per ml.
Ropy strains	0.2691 g.	1.52×10^9
Non-ropy Strains	0.2404	1.67×10^9

which it is apparent that no significant difference exists with respect to quantitative acetylmethylcarbinol production.

To test serological relationships among ropy and non-ropy strains of *Aerobacter aerogenes*, rabbit antiserum against 2 ropy strains was prepared, having a titer of 1:1280. Immunization was with three 0.5-ml. volumes at 5-day intervals, followed by two 1.0-ml. volumes at 5-day intervals, in the marginal ear vein. The antiserums were reacted against 10 aerogene cultures isolated from urinary infections. One culture failed to react. Nine others gave titers ranging from 1:40 to 1:160, indicating the presence of common antigens in ropy and non-ropy strains.

Staphylococcus cremoris-viscosus (Hammer) was conspicuous because of its ability to form strong rope and to digest milk completely in test tube culture in 24 hours at room temperature. This species was described in the fifth edition of Bergey's Manual *(3)* but was omitted from the sixth or 1948 edition. In view of the fact that no micrococcus resembling this organism appears in the sixth edition, this change would not seem to be justified.

Cultures of *Staphylococcus cremoris-viscosus* evidencing colonial roughness grew in litmus milk as a pellicle, with slime rather than ropiness apparent in the vicinity of the pellicle. Agar slants of such cultures often developed pigment varying from yellow to orange after a few days or weeks of storage.

DISCUSSION

The wisdom of omitting ropy strains of *Streptococcus lactis* from the preceding study may be questioned. Numerous studies of such cultures *(2, 5, 8, 10)* have been made from which the inference may be drawn that ropiness can develop spontaneously in lactic acid cultures. However, oral interrogation of 12 companies in the Pittsburgh, Pennsylvania, area revealed that never in their experience with lactic acid stock cultures, which extended back in some cases as far as 50 years, had they observed a culture to develop rope spontaneously. In the study just reported, ropy *Streptococcus lactis* cultures were isolated but eventually lost the ability to

produce rope. Also, a culture of *Streptococcus lactis* var. *hollandicus* so-called was obtained from the Iowa State College but lost the ability to produce rope after a few transfers in litmus milk. It would appear from these observations that ropiness of lactic acid cultures is not a stable variant characteristic. This opinion is in support of the observations of Beck and Chase *(1)*.

The influence of temperature upon ropiness was investigated but will be included in a later report dealing with the influence of physical environment upon the production of ropy substance.

In the present study it was easily apparent that the ability to produce ropiness in milk was associated with smooth colony forms. In general, mucoid appearance was present, except with alcaligenes cultures, and the degree of colony mucoidness was directly correlated with the degree of ropiness in milk. Dissociation to rough colony forms with accompanying loss of ropy characteristic in milk was commonly observed in cultures stored several months without transfer. With or without loss of rope, there were changes during storage of the indole, gelatin, nitrate, H_2S, and fermentative reactions, as well as appreciable changes in microscopic and colonial appearance. These changes, observed in all 3 species, were such as to raise doubt that they were the same cultures as those originally isolated. However, experience proved that variation in ropy milk cultures was not the result of errors or mishandling, but was an inevitable, normal occurrence.

SUMMARY

A study was made of bacteria capable of producing ropy milk, i.e., a state of high viscosity or plastic flow. Three stable, ropy species were identified in 440 cultures isolated from 3,500 samples of raw milk. The species and the most conspicuous characteristics of each as observed in this study are given below.

Alcaligenes viscous (Adametz) is a pleomorphic Gram-variable rod producing ropiness and most commonly an alkaline reaction in milk, but without the ability of fermenting sucrose.

Ropy *Aerobacter aerogenes* strains are indistinguishable in other respects from non-ropy strains isolated from sources other than milk, as judged by microscopic, cultural, biochemical, and serological studies.

Staphylococcus cremoris-viscosus is easily recognized by the production of ropiness and rapid proteolysis in milk at about 25°C. This organism deserves to be considered a distinct species.

With respect to all of the above species, loss of smoothness or mucoid colony characteristic is accompanied by decrease or loss of the ability to produce ropiness in milk, and is most likely to occur during prolonged storage of cultures without transfer.

LITERATURE CITED

1. Beck, E. C., and Chase, R. E. Some experiments dealing with ropy milk. *Sci. Agri.*, 19, 488 (1938).
2. Beijerinck, M. W. The streptococci of ropy whey. *Bot. Zeitung*, 40, 778 (1891).
3. Breed, R. S., Murray, E. G. D., and Hitchens, A. P. Bergey's *Manual of Determinative Bacteriology*. 5th ed., 1936, Williams and Wilkens Co., Inc., Baltimore, Md.

4. Breed, R. S., Murray, E. G. D., and Hitchens, A. P. Bergey's *Manual of Determinative Bacteriology*. 6th ed., 1948, Williams and Wilkens Co., Inc., Baltimore, Md.
5. Gorini, C. Concerning the lactic acid bacillus. *Milchiw. Zentrbl.*, **42**, 1 (1913).
6. Hammer, B. W., and Buchanan, R. E. Slimy and ropy milk. Ia. Agri. Ex. Sta. *Res. Bull., 22* (1915).
7. Long, H., and Hammer, B. W. Studies of alkaligenes viscosus. *Ia. Sta. Col. J. Sci.*, **10**, 261 (1936).
8. Macy, H. *Streptococcus lactis* var. *hollandicus*. *Abst. of Bact.*, **6**, 18 (1922).
9. Stahle, G. L., and Werkman, C. H. Acetylmethylcarbinol in fermentation liquors. *Ia. Sta. Col. J. Sci.*, **10**, 205 (1936).
10. Weigmann, H. Slime bacteria in milk. *Milchzeitung*, **18**, 982 (1899).

ROPY MILK. II. THE INFLUENCE OF PHYSICOCHEMICAL ENVIRONMENT ON THE FORMATION OF ROPY SUBSTANCE[a]

DANIEL L. JONES[b]
Department of Biological Sciences, Duquesne University, Pittsburgh, Pennsylvania

(Manuscript received October 1, 1953)

In a previous report *(2)* the above investigator noted that cultures may spontaneously lose the ability to produce ropiness in milk. An obvious question is how and why do they produce ropy or viscous material?

In the study to be reported here, work was begun with a view of determining whether, in general, rope formation is in response to a particular environment or is independent of environment. It is probable that the formation of ropy substance is an incidental rather than a vital function of the cell.

EXPERIMENTAL METHODS AND RESULTS

The effect of temperature. Five cultures of each species were inoculated by wire loop into 10 ml. of litmus milk for incubation at varying temperatures, with testing at regular intervals until the appearance of rope. The temperatures and times of ropiness are summarized in Table 1. Regardless of the temperature at which the minimum time for ropiness was noted, the highest degree of viscosity observed after additional incubation was at room temperature, or about 25°C.

TABLE 1

Temperature and time of ropiness in milk

Species	4.4°C.	10°C.	20°C.	30°C.	37.5°C.
	days	*days*	*hrs.*	*hrs.*	*hrs.*
Staphylococcus cremoris-viscosus	6	4	11	9	8
Aerobacter aerogenes	5	3	11	9	8
Alkaligenes viscosus	4	2	12	15	no growth

Studies of surface tension. Ten cultures of each species were made in triplicate in pint bottles of sterile skim milk. Unsterile skim milk was reserved as a control. Both were initially tested for surface tension with a Du Nroy surface tensiometer and were found to have a tension of 50 dynes/sq. cm. Additional surface tension measurements over a period of several days revealed no appreciable difference between the control and the ropy samples, even though it appeared that ropy slime developed and was concentrated in the surface of the milk culture. There was a decrease during incubation of control and test samples to the extent of nearly 4 dynes/ sq. cm.

In a parallel experiment, all 3 species were cultured in triplicate in litmus milk adjusted to a low surface tension at the time of inoculation. Adjustment was with

[a] In part from a dissertation submitted in partial fulfillment of the requirements for the Master of Science degree at the Pennsylvania State College, State College, Pennsylvania.

[b] Present address: The Hektoen Institute for Medical Research of the Cook County Hospital, Chicago, Illinois.

Tween 80 to 45 dynes/sq. cm. and below. Cultures incubated at 40 dynes/sq. cm. grew slowly at room temperature but eventually produced characteristic rope.

The effect of osmotic pressure. Five cultures of each of the ropy species were subcultured in sterile litmus milk containing 5% and 10% NaCl and in a litmus milk control. At these salt concentrations which are near the upper limits of tolerance the 2 Gram-negative species were inhibited but produced rope in the 5% concentration, whereas the staphylococcus grew slowly and produced rope in 10% NaCl in milk.

Reaction of the medium. Three cultures of each species were inoculated into sterile litmus milk buffered at pH 5.5, pH 7.0 and pH 8.5 with mixed phosphates.

Staphylococcus cremoris-viscosus produced an acid curd but no proteolysis or ropiness at pH 5.5. At pH 8.5 there was slow digestion of the milk proteins and weak ropiness or viscosity as compared to pH 7.0.

Aerobacter aerogenes grew well and produced rope at all 3 pH concentrations.

Alkaligenes viscosus grew poorly and produced weak rope at acid pH, but was typical at neutral and alkaline pH.

The influence of oxidation-reduction potential. From the fact that ropiness is first apparent in the surface of the medium it appeared either that oxygen was necessary for the production of rope, or more probably that cells divide more rapidly near the surface and hence form more product. To determine the facts all 3 species were tested in triplicate in aerobic and in anaerobic incubations in milk. Aerobic culture was in wide bottom Fernbach flasks in shallow layers. For anaerobic cultures, milk and also thioglycollate (Difco) broth in deep tubes were heated in boiling water to drive off air and layered with paraffin oil prior to inoculation. After 72 hours, all 3 species in aerobic milk cultures produced a semi-solid mass. Anaerobic milk cultures did not have the usual surface slime but did exhibit moderate ropiness when a wire loop was immersed and withdrawn. Aerogenes culture in thioglycollate also exhibited ropiness.

The result of agitation. Gentle agitation or aeration of milk cultures during incubation delayed and in some instances prevented the appearance of ropiness, although in the latter increased viscosity was apparent. Semi-solid ropy cultures of each species in milk subjected to violent agitation on a mechanical shaker for 5 minutes were no longer ropy after agitation. This loss of viscosity appeared to be permanent, as it did not return after several days of reincubation.

Chemical composition of the medium. A group of 19 semisynthetic media were prepared from various combinations of the following ingredients: Difco nutrient broth, casein, milk albumen, and simple carbohydrates, namely, glucose, lactose, and sucrose. In addition, a synthetic medium having the following per cent composition was devised: $(NH_4)2HPO_4$, 0.15; KH_2PO_4, 0.10; $CaCl_2$, 0.15; $MgSO_4$, 0.10; glucose, or glycerol, 2.0; and tap water to 1,000 ml. with adjustment of pH to 7.0. Five strains of each of the 3 species were tested for growth in each of these 21 broth media.

Staphylococcus cremoris-viscosus produced detectable viscosity in nutrient broth, provided simple carbohydrate was not present, but was ropy only in media containing albumen. Growth did not occur in casein broth nor in the synthetic broth media.

Alkaligenes viscosus also required albumen to produce true ropiness. Cultures grew well in the synthetic medium if 0.2% asparagine was added and if a comparable amount of lactate was present, but no ropiness was produced.

Aerobacter aerogenes was ropy in any medium capable of supporting growth, including the synthetic medium with glycerol as a source of carbon.

The action of toxic chemicals. Because of the effects of LiCl on some enzyme systems and because of the possibility that this compound can induce variation in bacterial population, studies were made with varying concentrations in litmus milk. After 3 transfers at 24-hour intervals of 3 species, using 4 strains of each, the cultures were plated on yeast whey agar containing 3% added agar but no LiCl. The findings as compared to control cultures are summarized in Table 2.

In confirmation that non-ropy colonies were not in a temporary, reversible condition, they were recultured in sterile litmus milk without LiCl but after several transfers evidenced no tendency to produce rope.

TABLE 2

Colony appearance after LiCl-milk culture

Species	0.5% LiCl	1.0% LiCl	2.0% LiCl
Staphylococcus cremoris-viscosus	Stimulated, ropy	Inhibited, no ropiness	No growth
Aerobacter aerogenes	Stimulated, ropy	Stimulated, ropy	Decreased growth, mixed ropy and non-ropy colonies
Alkaligenes viscosus	No effect, ropy	Weak growth, decreased rope	No growth

DISCUSSION

A series of erroneous statements regarding ropy milk have been carried forward in bacteriology textbooks for more than a decade. Specific reference is made to those of Hammer *(1)* and of Salle *(3)* as quoted in part below.

"The ropy condition is due to the formation of capsular material . . . the slime is a carbohydrate gum, although some protein as well as carbohydrate appears necessary for its formation." (Salle)[c]

"Often only the cream is ropy and the milk beneath is normal. This is because of the oxygen requirements of certain of the causative organisms." (Hammer)[d]

"It is probable that gums are more commonly the cause of ropiness in milk than mucins; with certain organisms the gums produced appear to be galactans." (Hammer)[e]

The so-called capsule of ropy milk bacteria is most probably not a true capsule but rather a material formed by an exoenzyme, particularly with ropy alkaligenes and staphylococcal cultures which produce this material from protein. The material is probably held in place in young cultures by surface tension. Certainly, in cultures which have stood for 24 hours or more this faintly staining ropy material is seen as an irregular mass around and among the cells.

The statement that the ropy material is a carbohydrate gum has never been really tested and proven to be true. It is a generalization based on the study of other capsulated bacteria and possibly on the studies of Schardinger *(4)* who found oxalic and mucic acids present after acid hydrolysis of a culture. He did not isolate and purify the ropy material. It will be demonstrated in a study yet to be reported that no polysaccharide *per se* is formed by any of the 3 species in milk. With at least 2 of these 3 species, ropy milk is produced in a manner analogous to the action of *Bacillus mesentericus* on gluten to produce a ropy bread. Carbohydrate was not required by any of the 3 species and if present and utilized was a hindrance to rope formation, which is probably a protein sparing action.

Lastly, aerobic conditions are not necessary for rope formation, as has

[c] Page 508 of reference 3.
[d] Page 90 of reference 2.
[e] Page 94 of reference 2.

been demonstrated here. The cultures grow more slowly in the absence of air but rope is produced, provided the substrate is satisfactory for its formation. Ropiness is more likely to be found in the cream line because rising of the fat carries the bacteria to the surface and not because air is a necessity.

SUMMARY

A study was performed with 3 bacterial species producing rope in milk and classified as *Alkaligenes viscosus, Aerobacter aerogenes,* and *Staphylococcus cremoris-viscosus.* The effect of chemical and physical environment on the ability to produce ropiness in milk was determined and is reported below.

The maximum degree of ropy viscosity is produced at room temperature, approximately 25°C. With the exception of *Alkaligenes viscosus,* which grows poorly at 30°C. and above, ropiness is produced most quickly at the optimum temperature for growth, which is 37°C.

Low surface tension, high osmostic pressure, and extremes of acid or alkaline reaction do not prevent ropiness if growth can occur. The exclusion of air from incubated potentially ropy cultures does not prevent ropiness.

Gentle agitation during incubation delays but does not prevent the appearance of viscosity or ropiness. Violent agitation of semisolid ropy cultures permanently destroys ropiness, suggesting a gel-like structure.

Alkaligenes viscous and *Staphylococcus cremoris* produce rope in milk by exoenzymatic attack on milk albumen. In contrast, *Aerobacter aerogenes* is ropy in any medium in which growth can occur. To some extent lithium chloride appears capable of forcing the variation of ropy species to non-ropy forms.

LITERATURE CITED

1. Jones, D. L. Ropy milk. I. The biology of rope production. *Food Research* (this issue).
2. Hammer, B. W. *Dairy Bacteriology.* Third ed., 1948, J. Wiley and Sons, New York, N. Y.
3. Salle, A. J. *Fundamental Principles of Bacteriology.* Third ed., 1948, McGraw-Hill Co., New York, N. Y.
4. Schardinger, F. The slime producing bacillus isolated from water. *Cent. F. Bakt.,* **8**, 144 (1902).

ROPY MILK. III. CHEMICAL STUDIES OF ROPY SUBSTANCE

DANIEL L. JONES
The Hektoen Institute for Medical Research of the Cook County Hospital, Chicago, Illinois

(Manuscript received October 1, 1953)

In a previous report by the above writer *(3)*, evidence was presented in support of the conclusion that, in two of three bacterial species studied, ropiness in milk is the result of attack of milk albumen. A product of protein degradation rather than a synthesized polysaccharide, this albumen would appear to be the cause of ropiness in such a circumstance.

A third species, *Aerobacter aerogenes*, was found capable of growing and producing ropiness in a synthetic medium with ammonia and glycerol as sources of nitrogen and carbon. Consequently, the mechanism of formation and the nature of the viscous material appeared to be more complex. Whether such ropiness be due to the presence of a synthesized polysaccharide, as is generally assumed, or whether, as reported by Stark and Stark *(5)*, ropiness results from a bacteriophage which induces a ropy variant from a non-ropy parent, are questions which merit further investigation.

EXPERIMENTAL METHODS AND RESULTS

The effect of enzymatic proteolysis upon ropiness. Three representative strains of each species were selected and cultured in 100-ml. volumes of milk for one week at room temperature, at which time they were highly ropy and exhibited plastic flow. To each flask was added 10 mg. of papain in 100 mg. of cystine. All flasks were incubated for 24 hours at 37°C. at which time all ropiness had entirely disappeared.

The straw colored fluid remaining from each culture was filtered and treated with 70% concentration of ethyl alcohol. The precipitate which formed was removed by centrifuging, dissolved in water, and reprecipitated a total of 8 times. The purified precipitates from all species were then tested and found to be positive to Millon's reagent and the biuret test for protein. All were negative with Benedict's test for carbohydrate, but aerogenes cultures gave a transiently positive test with alpha naphthol (Molisch) reagent. Hydrolysis of the samples for 2 hours in a boiling water bath with 2N HCl, neutralization, and repeating of carbohydrate qualitative tests gave the same result as before hydrolysis. A test for mucic acid (galactose) was included and found to be negative.

The effect of protein precipitation upon ropiness. The same 9 representative strains used above were cultured in 200-ml. volumes of milk for one week at room temperature. The cultures were combined to give a total of 600 ml. of highly ropy milk for each species. To 50-ml. aliquotes, inorganic protein precipitants, e.g. tungstic acid, were added. The water-clear filtrates gave only a mineral precipitate with ethyl alcohol. Also, protein precipitation with 10% trichloroacetic acid, centrifuging, and adding of 95% ethyl alcohol to the supernatant produced no precipitate in any of the samples.

In a final attempt to free protein from the supposed polysaccharide, ethyl alcohol was added to ropy milks at pH 5.0 to 30%, at which time a precipitate began to form and was complete at 40% alcohol concentration. The precipitate was removed by centrifugation. The remaining liquid was water clear and syrupy in appearance. Upon the further addition of alcohol to 70%, a second precipitate was obtained and due to its flocculent nature was removed by filtration. The precipitate from each of the 3 collective species was purified by 8 precipitations with alcohol. At this time, solutions of the purified "polysaccharide" gave a positive test for biuret linkage but negative qualitative tests for carbohydrate. The samples were again hydrolyzed with acid, neutralized, and tested qualitatively for carbohydrate. A weakly positive test was

obtained with the Molisch reagent from the aerogenes culture product. All other qualitative tests for carbohydrates were negative.

Tests for pentose were not included in the preceding work, as it has been generally assumed that a polymer of galactose or some other monosaccharide was associated with ropiness. By this time, it had become quite apparent that these assumptions were untrue. The important contributory substance appeared to be protein and only in the case of aerogenes was there a possibility of carbohydrate also being present. This carbohydrate, if present, gave a transiently positive test with Molisch reagent, but the Moore, Barfoed, and Benedict's tests were negative before and after acid hydrolysis of the supposed polysaccharide material.

Studies of Aerobacter aerogenes in synthetic media. To determine more accurately the nature of the ropy substance produced by *Aerobacter aerogenes*, and to eliminate interference from milk constituents, all subsequent studies were with this organism grown in the $(NH_4)2HPO_4$-glycerol-mineral supplement media previously described. Three strongly ropy cultures were selected and cultured in duplicate liter volumes at room temperature for one week. At this time, the liquid was a thick syrupy material exhibiting plastic flow. Bacterial proteins were removed by extraction, according to the method of Sevog *(4)*. The remaining liquid was free of noticeable viscosity and was condensed in volume by evaporation at 56°C. Qualitative tests for protein were negative. A dense white precipitate was obtained upon the addition of methyl alcohol to 70%. This precipitate was reprecipitated 5 times for purification and was again found to be free of protein by qualitative tests. A positive Bial's orcinol test for pentose was obtained but all other tests for carbohydrate were negative. The sample was heated with 5N HCl for one hour in a boiling water bath, neutralized and again tested for carbohydrates, with the same result. Upon analysis for inorganic phosphate by the method of Fiske and Subbarow *(2)*, a strongly positive test was obtained.

Proceeding on the assumption that a nucleic acid complex and not a polysaccharide was involved in the production of rope, the preceding experiment was repeated with minor modifications. The liter flasks of ropy milk were autoclaved with 1N HCl for 45 minutes at 15 pounds' pressure. Again the ropy characteristic disappeared as a result of hydrolysis. Melanins were observed to be present and were removed by precipitation with NaOH. After again condensing the solution, a test for purine was made with ammoniacal $AgNO_3$ and a positive test, consisting of a mixture of deep violet and brown color, was obtained. The carbohydrate was purified by 6 methyl alcohol precipitations and again gave a positive test for pentose and phosphorus. The test for purine was also positive but required 2 hours to develop.

To test whether *Alkaligenes viscosus* and *Staphylococcus cremoris-viscosus* would produce nucleic acid constituents in detectable quantity, these organisms were cultured in 1% tryptose broth. No viscosity was noted. Removal of proteins by the Sevog method of extraction, concentration at 56°C. and negative qualitative tests for purine and pentose, indicated that nucleic acid if present was in infinitesimal amount not detectable by qualitative test. Further support for this belief was given by the fact that addition of methyl alcohol to 75% in the sample protein-free produced only a faint non-filterable opalescence.

DISCUSSION

The action of *Alkaligenes viscosus* and *Staphylococcus cremoris-viscosus* upon milk albumen to produce ropiness seems to be rather clear-cut and quite analogous to the action of *Bacillus mesentericus* on gluten to produce ropy bread, as reported by Bunzell and Forbes *(1)*. However, the nature of ropiness in milk by *Aerobacter aerogenes* is not so clear-cut and work remains to be done for a complete understanding of the process. In an interesting study of ropy aerogenes by Stark and Stark *(5)* ropiness in milk was described as having been produced by the addition of bacteriophage to non-ropy cultures, causing them to "dissociate" to a form capable of producing sliminess or ropiness in milk. In the present study, the failure to find a true polysaccharide, and the abundance of nucleic acid constituents which were not found with the other 2 species would seem to

lend credence to the belief that ropiness in milk as produced by aerogenes cultures is somehow directly related to the synthesis of nucleoprotein, and possibly could result from infection by a bacteriophage. However, from colonial observations, no evidence of dissociation or lysis was physically apparent. Colonies were smooth, mucoid, and entire. When variation to non-mucoid forms was forced with liCl in a previous study, ropiness was lost. One would hesitate to say unequivocally that ropiness in milk cultures of aerogenes was due to the synthesis and presence of abundant amounts of nucleoprotein. Yet, the evidence in the present study points to that conclusion.

SUMMARY

In a study of *Alkaligenes viscosus* and *Staphylococcus cremoris-viscosus* in milk, ropiness was found to result from attack of albumen. No evidence of polysaccharide synthesis was found by definitive chemical techniques.

In a study of *Aerobacter aerogenes* cultures grown in synthetic medium and producing high viscosity therein, it was observed that destruction or removal of synthesized protein was accompanied by a loss of viscous characteristic. Furthermore, and in contrast to negative findings in semi-synthetic medium with the preceding two species, abundant amounts of nucleic acid constituents were found. Therefore, it would appear that the formation of rope in milk by this species is inseparably related to the synthesis of a nucleoprotein. The mechanism of formation, quite possibly by development of sub-lethal bacteriophage, has not been proven.

LITERATURE CITED

1. Bunzell, H. H., and Forbes, M. *Bacillus mesentericus*. *Cereal Chem.*, **7**, 465 (1930).
2. Fiske, C. H., and Subbarow, Y. The colorimetric determination of phosphorus. *J. Biol. Chem.*, **66**, 375 (1925).
3. Jones, D. L. Ropy milk. II. The influence of physicochemical environment on the formation of ropy substance. *Food Research* (this issue).
4. Sevog, M. S. Removal of denatured bacterial proteins. *Biochem. Ztschr.*, **273**, 419 (1934).
5. Stark, C. N., and Stark, P. Bacteriophage as a precursive agent in the development of organisms causing ropy milk. *J. Bact.*, **23**, 59 (1932).

COMPARISON OF THE NUTRITIVE VALUES OF MONO-, DI-, AND TRIGLYCERIDES BY A MODIFIED PAIR-FEEDING TECHNIQUE[a,b]

ROBERT S. HARRIS AND HENRY SHERMAN
Department of Food Technology, Massachusetts Institute of Technology, Cambridge, Massachusetts

(Manuscript received October 10, 1953)

It is many years since mono- and diglycerides were shown by Lewkowitsch *(8)* to be normal constituents of certain natural fats and oils. The great interest in these simpler glycerides which has developed during recent years is due primarily to the fact that they are being used extensively in manufactured shortenings and margarines *(9)*, and that they are formed as intermediate compounds during the digestion of triglycerides by higher animals and by man *(2, 3, 5, 10, 13)*.

Tidwell *(12)* reported that the monoglycerides of olive oil fatty acids were absorbed by rats at a faster rate than olive oil alone or fatty acids alone. Ames *et al. (1)* concluded that there is no significant difference in the growth response, reproductive ability, or lactation performance of rats fed the monoglycerides of cottonseed oil as compared with cottonseed oil. These two lipids were observed to have the same coefficient of absorption.

Mattson *et al. (9)* fed mono-, di-, and triglycerides of the same fatty acid composition to weanling rats *ad libitum* as 25% of a synthetic diet, and reported that, except for slight inherent differences in calorie values, these three lipids were nutritionally equivalent. When their data were calculated in terms of caloric efficiency (gain in body weight per 100 calories of diet eaten) the weight increases were identical.

It is likely that growth response to food calories is not an arithmetic function. Furthermore, the nutrient intake of animals fed diets differing in caloric value is not the same. The present series of experiments was designed to meet these objections, for rats of comparable groups were fed the same intake of food calories day by day and the intake of nutrients was essentially the same.

In the pair-feeding method, individual animals are offered a test diet *ad libitum*, and comparable animals are fed a control diet in a restricted amount. Since this method is useful only when the animals have a greater appetite for the control diet than the test diet, the animals on the control diet exist in a condition of mild starvation during the test period. The pair-feeding procedure has been criticized on this account. It is quite possible that the comparison would be more accurate if both the animals on the test and those on the control diets were pair-fed at levels which are less than their desires.

---

[a] Publication No. 174 from the Department of Food Technology, The Massachusetts Institute of Technology, Cambridge.

[b] Presented before the Thirteenth Annual Meeting of the Institute of Food Technologists, Boston, June 23, 1953.

The pair-feeding technique cannot be used to compare two diets which are equally appetizing to the test animals, for the *ad libitum* ingestion of the test diet sometimes exceeds that of the animals on the control diet. It is possible, however, to conduct a pair-feeding experiment on diets with similar appetite appeal by restricting slightly the food intake of the test group, and pair-feed the control diet at that level. In this paper this procedure will be referred to as the "restricted pair-feeding technique."

The restricted pair-feeding was carried out on a group rather than on an individual rat basis. Since the animals were carefully randomized between all groups there was no significant difference in the *ad libitum* requirements of the groups within a series and the comparisons are valid.

In the three series of experiments reported here, weanling rats were offered mono-, di-, and triglycerides and a mixture of these three fats, in a synthetic-type diet at approximately 15% and 25% levels, and in a diet of natural foodstuffs at approximately 15% levels. Within each series the fatty acid content of each diet per 100 calories was the same and the total calories offered each day to each group within each series were identical. Moreover, the protein/calorie ratio of the diets was the same in each experimental series.

EXPERIMENTAL

Preparation and characterization of diet fats. The triglycerides were prepared [c] from a mixture of partially hydrogenated soybean and cottonseed oils. The mixture

[c] The fats used in this study were prepared under the direction of Dr. F. V. Baur, Chemical Division, The Procter & Gamble Co., Cincinnati, Ohio.

TABLE 1

Characteristics of test fats [d]

	Monoglycerides	Diglycerides	Triglycerides	Mixture of mono-, di-, triglycerides
Glycerides				
Complete melting point	54.2	48.6	47.0	47.6
Saponification value	160.2	181.8	191.1	174.4
Acid value	9.9	1.7	<0.1	4.7
Hydroxyl value	303.0	95.0	5.0	165.0
Iodine value	62.3	71.7	74.8	68.1
Peroxide value (freshly opened cans)	0.0	0.10	0.51	0.28
Free glycerol	1.26%	0.03%	0.01%	0.71%
Monoglycerides	86.4	3.5	0.9	32.8
Diglycerides	6.6	92.3	2.2	52.8
Triglycerides	0.7	3.3	96.9	11.3
Free fatty acids	5.0	0.9	<0.1	2.4
Total fatty acids	79.3	90.7	95.5	86.6
Fatty acid composition				
Saturated acids	18.1%	17.4%	18.0%	17.8%
Oleic acid	76.8	77.5	77.0	77.1
Linoleic	4.9	4.8	4.7	4.9
Linolenic	0.04	0.06	0.06	0.05
Conjugated dienoic acids	0.20	0.21	0.19	0.18
Conjugated trienoic acids	....	<0.01	....	....

[d] Data supplied by the Technical Committee of the Institute of Shortening and Edible Oils, Inc.

TABLE 2

Composition of diets

	Experiment A				Experiment B				Experiment C			
Diet Group	224	225	226	227	228	229	230	231	232	233	234	235
Components	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.
Casein	26.2	26.8	27.0	26.6	26.2	26.8	27.0	26.6				
Sucrose	40.2	41.3	41.5	41.0	50.6	51.4	51.5	51.1				
Soybean oil	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0				
Salt mixture (6)	3.9	3.9	4.0	3.9	3.9	3.9	4.0	3.9				
Celluflour	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0				
Vitamin mixture*	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5				
Skim milk powder									21.6	21.6	21.6	21.6
Whole wheat									55.60	57.00	57.40	56.50
Meat powder (71% protein)									4.0	4.0	4.0	4.0
NaCl									1.2	1.2	1.2	1.2
$CaCO_3$									0.77	0.77	0.77	0.77
Vitamin A									1200IU	1200IU	1200IU	1200IU
Vitamin D									120IU	120IU	120IU	120IU
α-tocopherol												
Monoglycerides	27.2				16.8				16.8			
Diglycerides		25.5				15.4				15.4		
Triglycerides			25.0				15.0				15.0	
Mixture of mono-, di-, and triglycerides				26.0				15.9				15.9
Calories/100 g	509.1	521.0	524.6	517.3	464.2	469.8	471.4	468.5	416.9	421.4	423.0	420.4

*Vitamin mixture (mg./100 g. diet): thiamine HCl, 0.4; riboflavin, 0.5; pyridoxine HCl, 0.4; niacin, 2.0; Ca pantothenate, 2.0; inositol, 200.0; biotin, 0.03; folic acid, 0.25; choline chloride, 300.0; ascorbic acid, 10.0; menadione, 0.3; α-tocopherol, 10.0; p-aminobenzoic acid, 10.0; also 1250 USP units vitamin A and 125 USP vitamin D/100 g. diet.

of mono-, di-, and triglycerides was prepared from this hydrogenated triglyceride by interesterification at 180°C. with glycerol using sodium hydroxide as a catalyst *(4)*. The monoglycerides and diglycerides were prepared from the mixture of mono-, di-, and triglycerides by molecular distillation *(7)*. The characterizations of these 4 fats are given in Table 1.

Experimental procedure and composition of the diets. Weanling albino rats of the Sprague-Dawley strain were distributed into 12 groups of 15 rats each so that the average and the mean weights of each group were the same. The groups of animals were offered distilled water and the experimental diets outlined in Table 2.

In Experiment A the 4 test fats were fed at approximately 25% levels in a synthetic-type diet which was rich in high quality protein and contained liberal amounts of all minerals and vitamins known to be required in rat nutrition; in addition, other nutrients which are not required by rats when living on natural diets were included. At the beginning, each of the 4 groups was offered approximately 90% of its *ad libitum* demands as determined by a preliminary trial on a diet (No. 226) fed to rats of similar age. As the experiment progressed various groups failed to consume all the diet offered during each 24-hour period, and it was necessary to change the daily feeding levels to 80%, 70%, and finally 75% of the estimated *ad libitum* requirement. During the 70 days of this experiment each group consumed essentially all of the diets offered. Account was taken of the small differences in the calorie content of each diet and feedings were conducted on an equi-caloric basis, rather than on an equi-weight basis.

In Experiment B the 4 fats were fed at approximately 15% levels in the same synthetic-type diet as was fed in Experiment A. The initial feeding level was at 90% of the expected *ad libitum* demand; later the feedings were adjusted to 80%, to 75%, and then to 80% of this level. Feedings to the 4 groups were conducted on an equi-caloric basis.

In Experiment C the 4 fats were fed at approximately 15% levels in a natural diet similar to the Sherman B diet *(11)* but modified by replacing the whole milk powder by equivalent amounts of skim milk powder and test fats. These diets were first offered at a level estimated to be 90% of the *ad libitum* demand of weanling rats. Later it was changed to 75%, to 80%, and finally to 75% of *ad libitum* demand. All 4 groups were fed on an equi-caloric basis day by day.

TABLE 3

Summary of body weight and food consumption data

Diet No.	Diet characteristic	Rats/group	Weight gain/rat	Length of tibiae	Diet consumed /rat/day	Calories consumed /rat/day	Wt. gain /calorie consumed
	Experiment A. 25% test fats in synthetic-type diet						
			gm.	*mm.*	*gm.*	*cals.*	*gm.*
224	Monoglycerides	15	214 ± 3	39.1 ± 0.1	10.79	54.9	0.056
225	Diglycerides	15	211 ± 2	39.1 ± 0.1	10.43	54.3	0.055
226	Triglycerides	15	207 ± 2	38.8 ± 0.2	10.49	55.0	0.054
227	Mixture of mono-, di-, triglycerides	15	214 ± 2	38.9 ± 0.1	10.64	55.0	0.056
	Experiment B. 15% test fats in synthetic-type diet						
228	Monoglycerides	15	219 ± 2	38.7 ± 0.1	11.84	55.0	0.057
229	Diglycerides	15	220 ± 2	38.9 ± 0.2	11.60	55.1	0.058
230	Triglycerides	15	219 ± 2	38.9 ± 0.1	11.69	55.1	0.057
231	Mixture of mono-, di-, triglycerides	15	221 ± 3	39.0 ± 0.1	11.64	54.5	0.058
	Experiment C. 15% test fats in natural diet						
232	Monoglycerides	15	200 ± 2	37.8 ± 0.1	11.99	49.9	0.057
233	Diglycerides	15	201 ± 2	38.4 ± 0.2	11.78	49.6	0.058
234	Triglycerides	15	202 ± 2	38.4 ± 0.1	11.76	49.7	0.058
235	Mixture of mono-, di-, triglycerides	15	201 ± 2	37.9 ± 0.1	11.74	49.4	0.058

In all 3 experiments the rats were housed individually in wire mesh cages with raised flooring. The diet to be fed each group was divided equally between the 15 rats in the group and placed in feeding jars which were designed to minimize scattering. The food remaining in the feeding jars, and the small amount that was scattered during each 24-hour period were weighed. An amount of fresh diet equivalent to that scattered and uneaten was added to the ration to be fed during the following 24-hour period. Since very little food was scattered and since the several groups were fed less than their desires, this carry-over feeding of the diets proved to be an unimportant refinement in procedure. All animals were weighed twice weekly.

At the end of 70 days the rats were autopsied, a tibia from each rat was photographed by x-ray and the length of the tibia was measured on the photographic negative.

DISCUSSION

The data in Table 3 indicate that there were no significant differences in the increases of the body weights of groups fed the different fats in the same diets on an equi-caloric basis, under conditions of slightly restricted food intake. This observation was independent of the type of diet (natural or "synthetic") or the quantity of fat (15% or 25%) fed in the "synthetic" diet.

The average lengths of the tibiae of the rats receiving these diets were approximately the same, indicating that the skeletal size of the rats in each group was the same. The tibiae of the rats fed the natural diets were shorter than the tibiae of the rats fed the synthetic type diets. This would be expected for the animals on the natural diet consumed approximately 10% less calories and gained less body weight during the experiment.

The restricted pair-feeding technique employed in these experiments was designed to meet the most serious criticism which has been leveled against the original pair-feeding procedure. The groups in each series were maintained in a state of mild hunger which was essentially the same in all groups.

The animals in these 3 series of experiments showed a weight increase which was 60-70 g. less than was observed in a preliminary experiment with rats on the same diets fed on an *ad libitum* basis for 70 days. The comparisons within each experimental series are valid, nevertheless, because all groups received the same treatment.

SUMMARY

Male weanling rats were fed mono-, di-, and triglycerides and a mixture of these fats as 15% and 25% of a synthetic-type diet, and as 15% of a diet of natural foods, for 70 days on an equi-caloric, restricted, pair-feeding basis.

No significant difference in body weight increase or caloric-efficiency was observed and these fats appear to be nutritionally equivalent, calorie for calorie.

LITERATURE CITED

1. Ames, S. R., O'Grady, M. P., Embree, N. D., and Harris, P. L. Molecularly distilled monoglycerides. III. Nutritional studies on monoglycerides derived from cottonseed oil. *J. Am. Oil Chemists' Soc.*, **28**, 31 (1951).
2. Borgström, B. On the action of pancreatic lipase on triglycerides *in vivo* and *in vitro*. *Acta physiol. Scandinavica* **25**, 328 (1952).

3. DESNUELLE, P., AND CONSTANTIN, M. J. Formation de glycérides partiels pendant la lipolyse des triglycérides dans l'intestin. *Biochem. et Biophysica Acta*, **9**, 531 (1953).
4. EDELER, A., AND RICHARDSON, A. S. Process for manufacturing fatty esters. U. S. Patent 2,206,168 (1940).
5. FRAZER, A. C., AND SAMMONS, H. G. The formation of monoglycerides and diglycerides during the hydrolysis of triglycerides by pancreatic lipase. *Biochem. J.* **39**, 122 (1945).
6. HUBBELL, R. B., MENDEL, L. B., AND WAKEMAN, A. J. A new salt mixture for use in experimental diets. *J. Nutrition*, **14**, 273 (1937).
7. KUHRT, N. H., WELCH, E. A., AND KOVARIK, F. J. Molecularly distilled monoglycerides. I. Preparation and properties. *J. Am. Oil Chemists' Soc.*, **27**, 310 (1950).
8. LEWKOWITSCH, J. The meaning of the acetyl value in fat analysis. *Analyst*, **24**, 319 (1899).
9. MATTSON, F. H., BAUR, F. J., AND BECK, L. W. The comparative nutritive value of mono-, di-, and triglycerides. *J. Am. Oil Chemists' Soc.*, **28**, 386 (1951).
10. MATTSON, F. H., BENEDICT, J. H., MARTIN, J. B., AND BECK, L. W. Intermediates formed during the digestion of triglycerides. *J. Nutrition*, **48**, 335 (1952).
11. SHERMAN, H. C., CAMPBELL, H. L., AND RAGAN, M. S. Analytical and experimental study of the effects of increased protein with liberal calcium and riboflavin intakes: complete life cycles. *J. Nutrition* **37**, 317 (1949).
12. TIDWELL, H. C. Mechanism of fat absorption as evidenced by chylmomicrographic studies. *J. Biol. Chem.*, **182**, 405 (1950).
13. VERZAR, F., AND MCDOUGALL, E. J. *Absorption from the Intestine*. 1936, Longmans, Green and Co., New York, N. Y.

DIFCO

preferred media
for isolation and differentiation
of

ENTERIC PATHOGENS

SALMONELLA — SHIGELLA

Isolation

Bacto-S S Agar
Bacto-MacConkey Agar
Bacto-Bismuth Sulfite Agar
Bacto-Brilliant Green Agar
Bacto-Selenite Broth
Bacto-Tetrathionate Broth Base

Differentiation

Bacto-Triple Sugar Iron Agar
Bacto-S I M Medium
Bacto-Purple Broth Base
Bacto-Purple Agar Base
Bacto-Urea Broth
Bacto-Urea Agar Base

ENTEROCOCCI

Bacto-Azide Dextrose Broth
Bacto-Azide Blood Agar Base
Bacto-S F Medium
Bacto-Phenylethanol Agar
Bacto-Enterococci Presumptive Broth
Bacto-Enterococci Confirmatory Broth
Bacto-Enterococci Confirmatory Agar

ENDAMOEBA HISTOLYTICA

Bacto-Endamoeba Medium
with Bacto-Horse Serum and Bacto-Rice Powder

THE DIFCO MANUAL, *NINTH EDITION*,
including descriptions of these media and their use,
is available on request.

Specify DIFCO — the trade name of the pioneers in the research and development of Bacto-Peptone and Dehydrated Culture Media

DIFCO LABORATORIES
DETROIT 1, MICHIGAN